

Induction of indirect plant defense in the context of multiple herbivory

Gene transcription, volatile emission, and predator
behavior

Tila R. Menzel

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Thesis

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Abstract

Plants live in complex environments and are under constant threat of being attacked by herbivorous arthropods. Consequently plants possess an arsenal of sophisticated mechanisms in order to defend themselves against their ubiquitous attackers. Induced indirect defenses involve the attraction of natural enemies of herbivores, such as predators and parasitoids. Predators and parasitoids use odors emitted by damaged plants that serve as a “cry for help” to find their respective prey or host herbivore. The aim of this thesis was to use a multidisciplinary approach, with focus on molecular and chemical methods, combined with behavioral investigations, to elucidate the mechanisms of plant responses to multiple herbivory that affect a tritrophic system consisting of a plant, an herbivore and a natural enemy.

Induced plant defenses are regulated by a network of defense signaling pathways in which phytohormones act as signaling molecules. Accordingly, simulation of herbivory by exogenous application of phytohormones and actual herbivory by the two-spotted spider mite *Tetranychus urticae* affected transcript levels of a defense gene involved in indirect defense in Lima bean. However, two other genes involved in defense were not affected at the time point investigated. Moreover, application of a low dose of JA followed by minor herbivory by *T. urticae* spider mites affected gene transcript levels and emissions of plant volatiles commonly associated with herbivory. Only endogenous phytohormone levels of jasmonic acid (JA), but not salicylic acid (SA), were affected by treatments. Nevertheless, the low-dose JA application resulted in a synergistic effect on gene transcription and an increased emission of a volatile compound involved in indirect defense after herbivore infestation.

Caterpillar feeding as well as application of caterpillar oral secretion on mechanically inflicted wounds are frequently used to induce plant defense against biting-chewing insects, which is JA-related. Feeding damage by two caterpillar species caused mostly identical induction of gene transcription, but combination of mechanical damage and oral secretions of caterpillars caused differential induction of the transcription of defense genes. Nevertheless, gene transcript levels for plants that subsequently experienced an infestation by *T. urticae* were only different for a gene potentially involved in direct defense of plants that experienced a single event of herbivory by *T. urticae*. Indirect defense was not affected. Also sequential induction of plant defense by caterpillar oral secretion and an infestation by *T. urticae* spider mites did not interfere with attraction of the specialist predatory mite *P. persimilis* in olfactometer assays. The predator did distinguish between plants induced by spider mites and plants induced by the combination of mechanical damage and caterpillar oral secretion but not between plants with single spider mite infestation and plants induced by caterpillar oral secretion prior to spider mite infestation. The composition of the volatile blends emitted by plants induced by spider mites only or by the sequential induction treatment of caterpillar oral secretion followed by spider mite infestation were similar. Consequently, the induction of plant indirect defense as applied in these experiments was not affected by previous treatment with oral secretion of caterpillars. Moreover, herbivory by conspecific *T. urticae* mites did not affect gene transcript

levels or emission of volatiles of plants that experienced two bouts of herbivore attack by conspecific spider mites compared to plants that experienced only one bout of spider mite attack. This suggests that Lima bean plants do not increase defense in response to sequential herbivory by *T. urticae*.

In conclusion, using a multidisciplinary approach new insights were obtained in the mechanisms of induction of indirect plant defense and tritrophic interactions in a multiple herbivore context, providing helpful leads for future research on plant responses to multiple stresses.

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Chapter 1

General Introduction

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General Introduction

The green leaf material of plants is exploited as a source of nutrients by other organisms. Arthropods, including insects, are the most species-rich group of organisms with an estimated six million species, of which approximately 50% are herbivorous (Schoonhoven *et al.*, 2005). Plants and insects co-exist since the Devonian Era from 416 million years ago to 359 million years ago (Labandeira, 2007). Despite extensive tissue damage and complete defoliation at times, plants are evidently able to persist and thrive. Their inability to escape their ubiquitous herbivorous attackers requires plants to rely on a combination of constitutive and induced defenses to protect them against insect herbivores (Schoonhoven *et al.*, 2005). Constitutive defenses comprise defensive structure such as thorns, waxy layers on plant tissues, and trichomes, and often provide a first line of defense. Induced defenses are a second line of defense, and are a highly plastic trait allowing plants to change their phenotype according to external stimuli in their environment that indicate the presence of herbivores, including the touch by an attacker (Bown *et al.*, 2002). Induced defenses include changes in morphology or synthesis of defense-associated proteins and secondary metabolites. The latter include for instance toxic compounds, proteinase inhibitors that interfere with digestion of plant material, and volatile organic compounds that repel herbivores or attract the natural enemies of herbivores (Mithöfer and Boland, 2012). Since defenses come at metabolic costs and ecological costs, plants have developed a sophisticated network of regulatory mechanisms to optimize their defense for maximum selectivity and efficiency (Karban *et al.*, 1997; Baldwin, 1998; Heil and Baldwin, 2002; Strauss *et al.*, 2002).

Early signaling steps in herbivore recognition

During the past years, advances in molecular, genetic, and biochemical methods have made it possible to identify some of the key players in signaling pathways leading to plant defense responses. The first step in the activation of any type of induced plant defense involves the sensing of the invader and recognition of an attack. Plants possess a complex sensory system which responds to mere touch by a herbivore, deposition of non-feeding stages such as eggs, and tissue damage caused and insect-derived elicitors introduced by feeding herbivores (reviewed in Hilker and Meiners, 2010). Cell membrane depolarization and increase in cytosolic Ca^{2+} which occurs within seconds, is an early event in defense signaling and appears to be a master regulator required for many subsequent signaling steps (Fig. 1) (Scheel, 1998; Maffei *et al.*, 2007). Next to Ca^{2+} and H^+ influx, Cl^- efflux, membrane depolarization and production of reactive oxygen species (ROS), such as H_2O_2 and nitric oxide (NO), also belong to the early events in generating plant defense (Levine *et al.*, 1994; Pugin, 1997; Blume *et al.*, 2000). Moreover, ROS, which accumulate in local and systemic leaves of attacked plants act thereby not only as signaling molecules but also as defense mechanism (Orozco-Cárdenas *et al.*, 2001; Foyer and Noctor, 2005).

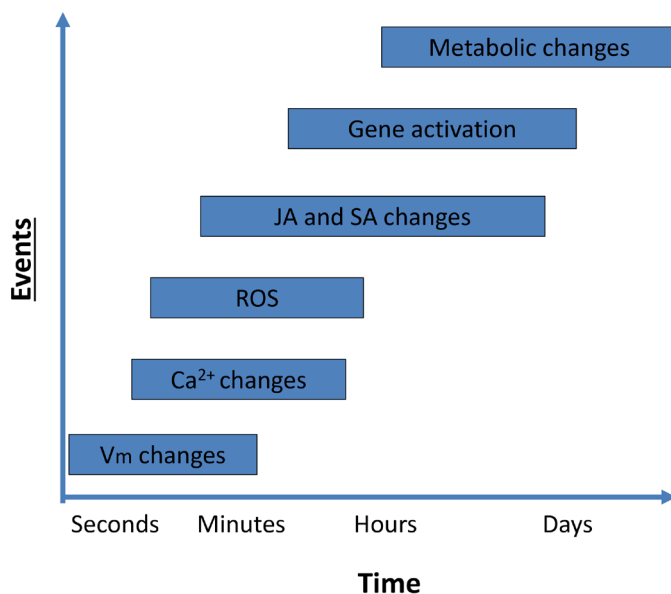


Fig. 1. Overview of events involved in the generation of an induced plant defense response in chronological order. Modified after Maffei *et al.* (2007). Abbreviations: Membrane potential (Vm), jasmonic acid (JA), salicylic acid (SA), reactive oxygen species (ROS)

Subsequently, Mitogen-activated protein kinases (MAPK) transfer information from sensors to cellular responses, and MAPK cascades are important pathways that play a role in signaling of plant hormones, as well as (a)biotic stresses, and pathogens (Maffei *et al.*, 2007; Pieterse *et al.*, 2012).

Phytohormone signaling and defense activation

Plant hormones, also known as phytohormones, act as central players in activating the actual plant defense signaling network leading to synthesis of defensive metabolites downstream of molecular recognition events. The two phytohormones jasmonic acid (JA) and salicylic acid (SA) are widely recognized as the major defense hormones, whereas other phytohormones, such as ethylene (ET), function rather as modulators of defense responses mediated by the two major defense hormones (Van Loon *et al.*, 2006; Browse, 2009; Pieterse *et al.*, 2012). Depending on the identity of an attacker, antagonistic and synergistic phytohormone pathways can be differentially activated, whereby composition and timing of the signaling pathways induced can determine whether plant tissues become more susceptible or resistant to stress. This so-called phytohormone crosstalk allows plants to regulate and fine-tune their defense responses in an attacker-specific manner in terms of activation of specific sets of defense genes and production of defensive chemical compounds (Reymond and Farmer, 1998; Koornneef and Pieterse, 2008; Thaler *et al.*, 2012). Chemically diverse compounds with a possible defensive function are produced by plants and belong to various chemical classes. The main chemical classes include for instance terpenoids, N-containing alkaloids,

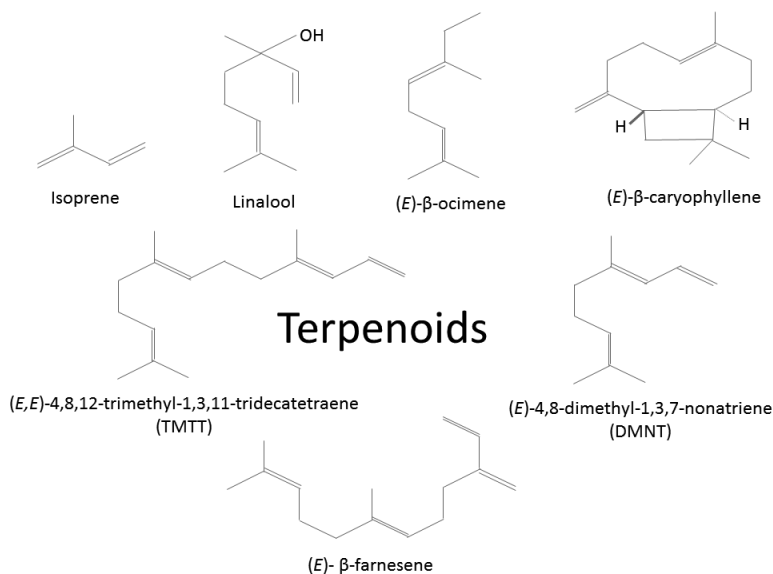
and phenolic compounds including flavonoids (Mithöfer and Boland, 2012). However, the majority of defense compounds are terpenoids, which comprise a large and diverse class of organic compounds, of which some 30,000 molecular structures have been identified and characterized. Whereas terpenoids are produced ubiquitously among plants, some defense compounds are produced only by certain plant taxa. Many defense compounds need to be stored in specialized compartments, such as vacuoles or the apoplast in order to avoid autotoxicity. The general mode of action of defensive compounds includes membrane disruption, inhibition of nutrient and ion transport, inhibition of signal-transduction processes, inhibition of metabolism, or disruption of hormonal control of physiological processes (Wittstock and Gershenzon, 2002; Mumm and Hilker, 2006; Mithöfer and Boland, 2012). However, not all chemical compounds involved in plant defense affect herbivorous attackers directly, and are thus part of the so-called plant indirect defense.

Plant indirect defense and involved compounds

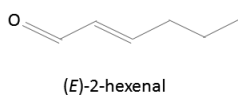
Some volatile organic compounds are released by plants and attract natural enemies of herbivores, such as predators and parasitoids. Since herbivorous arthropods are often small organisms compared to the host plant they consume and try to avoid betraying their presence, their natural enemies often rely on chemical information provided by plants, such as volatile compounds, to serve as reliable and detectable host-location cues. Dicke and colleagues (Dicke and Sabelis, 1988; Dicke *et al.*, 1990) were the first to provide evidence for an active release of VOCs in response to herbivory in order to attract natural enemies. Since then the actual complexity of these tritrophic interactions between plant, herbivore, and natural enemy has been the subject of many studies. Volatile blends released after herbivory may consist of up to 200 VOCs, also known as herbivore-induced plants volatiles (HIPVs), which can be roughly divided into 3 groups, namely terpenoids, fatty acid derivatives and phenylpropanoids or benzenoids (Fig. 2) (Mumm and Dicke, 2010).

The key players among terpenoid volatiles consist of monoterpenes (C₁₀), sesquiterpenes (C₁₅), and homoterpenes (C₁₁ or C₁₆), which all significantly contribute to any blend of plant-derived volatiles. All terpenoids are synthesized via one of two pathways, known as the cytosolic mevalonate (MVA) pathway and the methylerythritol 4-phosphate (MEP) pathway (Chappell, 1995; Aharoni *et al.*, 2005; Cheng *et al.*, 2007). Antagonistic or synergistic crosstalk between JA, SA, and ET signaling pathways has been suggested to regulate the characteristic blend of terpenoids in response to herbivory (Ozawa *et al.*, 2000; Engelberth *et al.*, 2001; Horiuchi *et al.*, 2001; Schmelz *et al.*, 2003). Moreover, it has been shown that terpenoids can be attractive to natural enemies themselves or act synergistically with other plant volatiles or herbivore pheromones (Dicke *et al.*, 1990; Erbilgin and Raffa, 2001; Pettersson, 2001; De Boer and Dicke, 2004; Mumm and Hilker, 2005).

Volatile fatty acid derivatives are often associated with the green leaf odor emitted after tissue damage and are also known as green leaf volatiles (GLVs). GLVs originate from C₁₈ unsaturated fatty acids, such as linoleic acid and linolenic acid, and are synthesized via the octadecanoid pathway through which also the phytohormone JA is produced.



Green leaf volatiles (GLVs)



Phenylpropanoids

or

Benzenoids

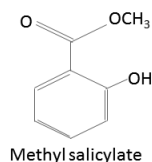
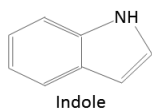


Fig. 2. Representative compounds of the three major classes of compounds found in the headspace of plants induced by herbivory or egg deposition. Adapted from Mumm and Dicke (2010).

In plant indirect defense they act as attractants for natural enemies of herbivores or take part as volatile signal involved in systemic induction or priming of changes in plant phenotype (Reddy, 2002; Shiojiri *et al.*, 2006; Frost *et al.*, 2007; Heil and Bueno, 2007; Van Wijk *et al.*, 2008; Wei and Kang, 2011).

Phenylpropanoids and benzenoids originate from the amino acid L-phenylalanine, but comparatively little is known about the detailed biosynthesis of these compounds (Dudareva *et al.*, 2006). The benzenoid ester methyl salicylate (MeSA) is the best studied of this class of compounds because it is frequently emitted by herbivore-infested plants and functions in plant indirect defense (Dicke *et al.*, 1990; Van Poecke and Dicke, 2002; James, 2003; Ament *et al.*, 2004; De Boer *et al.*, 2004).

Generally, plant volatiles, including the major classes of HIPVs, are low-molecular-weight compounds (below 300 Da) that are lipophilic liquids with high vapor pressure (Pichersky *et al.*, 2006). This way they are able to cross membranes freely to be released into the atmosphere or soil. Thereby they are not only released from attacked sites, but also from systemic tissues, and can be perceived by herbivores, natural enemies of herbivores, and neighboring plants. Herbivores may be attracted or repelled depending on the blend and neighboring plants may respond to the information by entering a state of increased readiness for a herbivore attack which is called the “primed state” (Bruin *et al.*, 1992; Bolter *et al.*, 1997; Arimura *et al.*, 2000; Kalberer *et al.*, 2001; Karban *et al.*, 2003; Conrath, 2009). Composition of volatile blends is influenced by underlying defense pathways induced by a herbivore, and a certain blend can convey information to other members of the surrounding ecological community. Information contained in a volatile blend includes, for instance, information on plant species, herbivore species, density, and developmental stage (Takabayashi *et al.*, 1995; De Moraes *et al.*, 1998; Gols *et al.*, 2003).

Specific activation of defense signaling pathways seems to be important in transmitting all this information. Generally, plant defense responses to wounding, biting-chewing herbivores, and certain cell-content feeding mites, are regulated by the JA signaling pathway (McConn *et al.*, 1997; Ament *et al.*, 2004). In contrast, plant defense responses against piercing-sucking herbivores and the generation of systemic acquired resistance (SAR) are regulated by the SA signaling pathway (Conrath *et al.*, 2001; Kempema *et al.*, 2007). However, cross-talk between the JA and SA signaling pathways, may take the form of antagonism and occur via MAPKs, transcription factors, or phytohormones, and thus allow for fine-tuning of plant defense responses against specific herbivores (Pieterse and Dicke, 2007; Thaler *et al.*, 2012; Stam *et al.*, 2014). Even though JA, SA, and ET are evidently important signaling molecules, additional layers of regulation seem to shape the outcome of defense responses. Yet, unidentified regulatory factors or cross-talk between the signaling pathways and the resulting differences in quantity, composition and timing of phytohormones might act in fine-tuning of plant defense responses and activate distinct sets of defense-related genes (De Vos *et al.*, 2005). Herbivore-associated elicitors (HAE) are likely to be involved in increasing specificity of plant defense responses (Bonaventure *et al.*, 2011). Exposure of plants to the combination of artificial wounding and HAE, as present for example in caterpillar oral secretions, has

been shown to induce defense responses that closely resemble herbivore feeding in plants (Turlings *et al.*, 1990). Moreover, Bidart-Bouzat *et al.* (2011) showed that even herbivores with the same feeding mode can induce species-specific defense responses in terms of gene transcription, and defense metabolite induction. The HAE identified to date comprise a large variety of molecules, including enzymes, modified forms of lipids, sulphur-containing fatty acids, fragments of cell walls, and peptides from digested plant proteins (Doares *et al.*, 1995; Mattiacci *et al.*, 1995; Alborn *et al.*, 1997; Bergey *et al.*, 1999; Schmelz *et al.*, 2006; Alborn *et al.*, 2007). It has been suggested that HAE are essential for growth and development of herbivores and thus comprise traits that are not easily lost over evolutionary time, despite the disadvantages they pose (Yoshinaga *et al.*, 2008).

Multiple herbivores and plant defense

Plant defense responses to a single biotic stress, such as attack by a single herbivore species, have been extensively studied for many plant-herbivore and tritrophic interactions. However, in their natural environment, plants live in complex communities consisting of neighboring plants and various arthropods from different trophic levels (Fig. 3). Changes caused in a plant phenotype by phytohormones and the underlying transcriptomic changes induced by herbivore feeding are thus able to affect the other members of the community directly or indirectly.

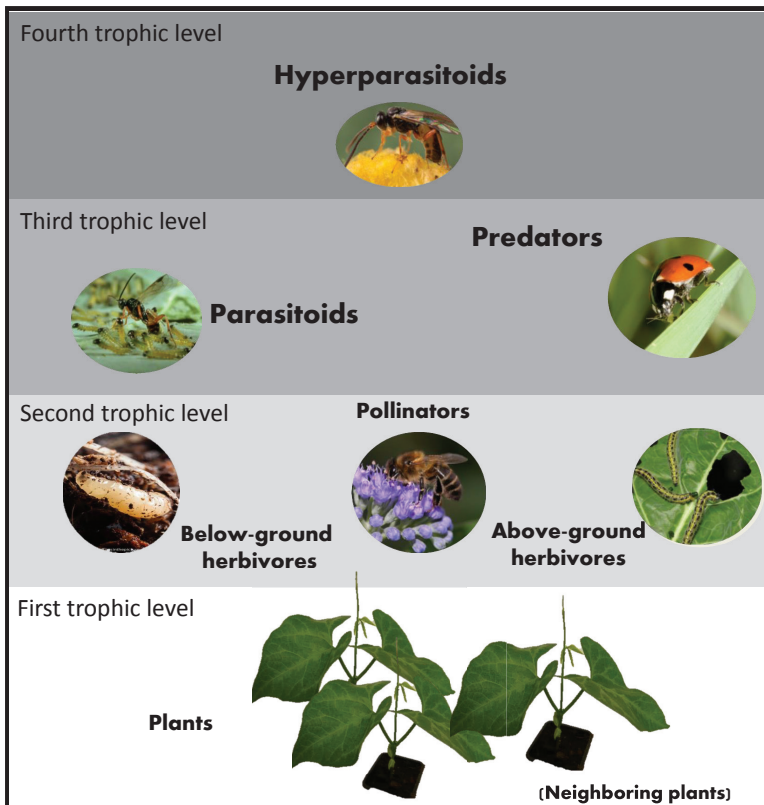


Fig. 3. Simplified overview of the members of the community associated with a plant. Adapted from Stam *et al.* (2014). Insect pictures are courtesy of Hans Smid and Nina Fatouros, www.bugsinthepicture.com

Indeed several studies have shown that herbivory by one species can affect the performance of a second herbivore species (e.g. Van Zandt and Agrawal, 2004; Viswanathan *et al.*, 2007; Poelman *et al.*, 2008; Erb *et al.*, 2011). Moreover, multiple herbivory can cause temporal changes in defense phytohormone signaling and the underlying gene regulatory network that subsequently lead to distinct defense responses. Some of these latter defense responses have shown to be in fact different than the combined response to either individual attacker (De Boer *et al.*, 2008; Rodriguez-Saona *et al.*, 2010). Higher trophic levels such as natural enemies of herbivores can be affected by phenotypic changes caused by interactions of multiple herbivores as well (Dicke *et al.*, 2009). Generally, herbivores which induce the same defense signaling pathways seem to increase the level of plant defense, including the attraction of natural enemies (De Boer *et al.*, 2008). In contrast, herbivores which induce antagonistic signaling pathways seem to interfere with plant indirect defenses (Zhang *et al.*, 2009). However, the effect of multiple herbivores on plant defense also depends on factors such as length of the time interval after which a second herbivore species attacks the same plant, on the sequence of attack and herbivore densities. Time intervals are important because plants are able to form a sort of memory, also known as “priming”, in response to herbivory, which can lead to increased defenses against subsequent herbivores (Frost *et al.*, 2008). Even plants that have not experienced herbivory themselves, have been shown to increase direct and indirect defenses against subsequent herbivores in response to exposure to HIPVs from attacked neighboring plants (Kost and Heil, 2006; Yi *et al.*, 2009; Peng *et al.*, 2011). It is still unclear how this mechanism of memory formation works, but involvement of phytohormones and accumulation of signaling proteins in their inactive form have been proposed (Conrath *et al.*, 2001; Beckers *et al.*, 2009).

Studies that probe the dynamics underlying regulatory modules of plant defense, including phytohormones and gene transcription, are necessary for a better understanding of how plants deal with complex interactions within their associated communities. The objective of this thesis is to study the underlying mechanisms of plant indirect defense in response to damage by different herbivores and multiple herbivory. The following research questions describe three existing gaps in our knowledge about tritrophic interactions in a multiple-herbivore environment, which will be addressed in this thesis:

Question I: Can plant indirect defense genes and metabolites be primed with low doses of phytohormones?

Question II: Does minor herbivory change or prime plant genes and metabolites for enhanced induction of indirect defense by subsequent herbivory?

Question III: Do herbivores that differ in feeding mode differentially influence plant indirect defense in terms of gene transcription and metabolites?

Study system

A tritrophic system consisting of Lima bean plants, two-spotted spider mites and predatory mites was used as the basis for studying the research questions of this thesis (Fig. 4).

Tetranychus urticae, the two-spotted spider mite, is a highly polyphagous agricultural pest that is distributed globally and is able to feed on a wide variety of over 960 different plant species (Fig. 4) (Jeppson *et al.*, 1975). The mites use their needle-like stylets to feed on the cell contents of plant parenchyma cells by piercing through cell walls. This feeding results in decreases in photosynthetic capability of plants, and causes changes in essential physiological processes such as transpiration, stomatal conductance and respiration (Landeros *et al.*, 2004; Reddall *et al.*, 2004). Fast growing populations are often founded by a single female which can lay up to 2 - 3 eggs per day on bean plants and have a lifespan of up to 30 days. The rate of development and oviposition are dependent on host plant and temperature. On bean plants, *T. urticae* completes its four-stage development from egg to adult in 13 - 21 days at 22 °C (Jeppson *et al.*, 1975). In the absence of natural enemies or other control measures, the quick population growth of spider mites results in rapid overexploitation of the host. Afterwards the mites disperse through the air using strands of silk.

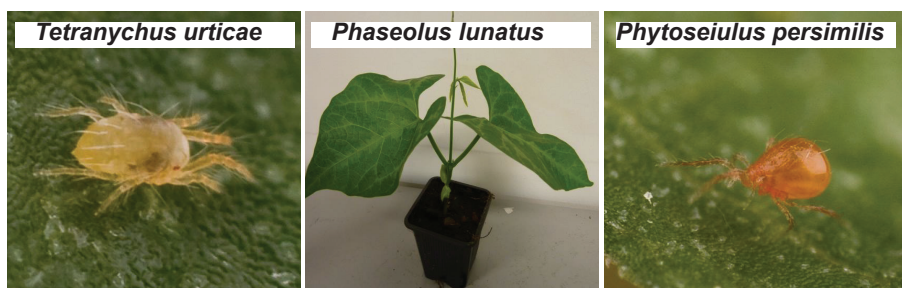


Fig. 4. Three species involved in the principal tritrophic interaction studied in this thesis (mite pictures courtesy of Hans Smid, www.bugsinthepicture.com). Average size of *T. urticae* females is approximately 0.4 mm and for *P. persimilis* females approximately 0.5 mm.

Phytoseiulus persimilis is a specialist predatory mite originating from South America, which is successfully used worldwide as biological control agent against *T. urticae* (Fig. 4). This voracious carnivore specializes on spider mites in the genus *Tetranychus*, and prefers to feed on eggs of their prey. One *P. persimilis* mite may consume up to 30 eggs per day (Sabelis, 1981). The blind predators use chemical cues, including HIPVs, as main prey-locating cues (Sabelis and Van De Baan, 1983; Dicke *et al.*, 1990). Just as for *T. urticae*, predator colonies are often founded by a single female, which can lay up to five eggs per day. Developmental time from egg to adult is temperature dependent and may take less than a week under favorable conditions. Once a prey population is eradicated minute wingless phytoseiid predators rely only on passive transport by wind currents for long-range dispersal (Johnson and Croft, 1976, 1981; Hoy, 1982).

The tritrophic interaction between Lima bean plants, the herbivores and natural enemies has been well studied. It is frequently used as a model system to study induced indirect defenses (e.g. Dicke *et al.*, 1999; Dicke *et al.*, 2003; Horiuchi *et al.*, 2003; Mithöfer *et al.*, 2005; Heil and Bueno, 2007; Mumm *et al.*, 2008). Induced indirect defense by Lima bean plants includes, for example, the secretion of extrafloral nectar (EFN) and the release of HIPVs. Both EFN and HIPVs are an inducible defense mechanism in the sense that their production rate increases in response to herbivory and their response is regulated by the herbivore-induced phytohormone signaling pathways (Hopke *et al.*, 1994; Dicke *et al.*, 1999; Heil *et al.*, 2001). Moreover, both defense mechanisms can be induced by exogenous JA application (Dicke *et al.*, 1999; Heil, 2004). EFN is produced by specialized nectar-producing glands which are located at the base of the petioles and thus physically apart from the flower. They provide a carbohydrate-rich exudate that is used as food by natural enemies of herbivores (Kost and Heil, 2005). In contrast, HIPVs are produced and released locally and systemically from herbivore-damaged sites and provide natural enemies and other plants with valuable information on host or prey presence. Consequently, EFN-related indirect defense in Lima bean has been mostly studied in the context of ants as bodyguards (e.g. Kost and Heil, 2005, 2006; Ballhorn *et al.*, 2014), whereas HIPVs have been investigated for their interaction with *P. persimilis* (e.g. Dicke *et al.*, 1990; Gols *et al.*, 2003; De Boer and Dicke, 2004; De Boer *et al.*, 2004; De Boer *et al.*, 2008; Zhang *et al.*, 2009) and other plants (e.g. Arimura *et al.*, 2000; Choh *et al.*, 2004; Heil and Bueno, 2007; Muroi *et al.*, 2011).

Herbivory by *T. urticae* is known to induce *de novo* synthesis of Lima bean plant volatiles which attract the predator *P. persimilis* (Dicke *et al.*, 1999). Moreover, neighboring plants have been shown to perceive these volatile signals and can become “primed” and in case of infestation respond with enhanced indirect defense (Choh *et al.*, 2004; Heil and Kost, 2006). Several studies have outlined the crucial role of the JA and SA signaling pathway in shaping indirect defense and the composition of herbivore-specific induced HIPV blends of different herbivores in Lima bean (Ozawa *et al.*, 2000; Zhang *et al.*, 2009; Wei *et al.*, 2014). Dicke *et al.* (1999) found that exogenous application of JA induces a volatile blend that is attractive to *P. persimilis* as it closely resembles the *T. urticae*-induced blend. Moreover, Gols *et al.* (2003) found that concomitant application of JA and *T. urticae* potentiated indirect defense. To date it is one of the few tritrophic interactions for which the principal attractive compounds that mediate the interaction with a natural enemy have been identified (Dicke *et al.*, 1990; De Boer and Dicke, 2004; De Boer *et al.*, 2004). Despite the fact that the Lima bean genome has not been sequenced yet, sequences of a limited number of genes potentially involved in Lima bean defense have been identified (Arimura *et al.*, 2000; Arimura *et al.*, 2008). Of particular interest in this context is the information about the *Phaseolus lunatus ocimene synthase* (*PIOS*) gene. *Ocimene synthase* is the enzyme that catalyzes the last dedicated step in the synthesis of (*E*)- β -ocimene. The monoterpene (*E*)- β -ocimene is an HIPV produced by many plant species in response to herbivory and has been identified as one of the principal attractants of *P. persimilis* (Dicke *et al.*, 1990). Other genes that have been identified include pathogenesis-related (PR) genes, such as the β -1,3-glucanase PR-2 and the chitinases PR-3 and PR-4, a lipoxygenase (LOX), which catalyzes an early step in the JA signaling pathway, phenylalanine ammonia-lyase (PAL) in the phenylpropanoid pathway (as part of

the SA signaling pathway), and farnesyl pyrophosphate synthetase (FPS) related to isoprene biosynthesis which is the precursor of terpenoid biosynthesis. Nevertheless, the number of studies aimed at relating indirect defense in terms of behavior, metabolites, phytohormones and genes for this system is quite limited (Arimura *et al.*, 2000; Ozawa *et al.*, 2000; Arimura *et al.*, 2002; Arimura *et al.*, 2008; Ozawa *et al.*, 2009; Zhang *et al.*, 2009). Here, I will integrate molecular, chemical and behavioral approaches to elucidate effects of multiple herbivory on tritrophic interactions through phenotypic changes of the plant.

Thesis outline

Chapter 2 explores how gene transcript levels of relevant defense genes which were used in the other chapters to monitor plant defense responses, are affected by phytohormones and herbivory. The effects of phytohormone dose and herbivore density were assessed.

In Chapter 3 the question whether a low-dose JA application can induce priming of plant indirect defense, and thus memory formation, was addressed. This was done in a multidisciplinary approach that included the quantification of endogenous phytohormone levels, gene transcript levels of relevant defense genes, and the resulting blend of volatile metabolites.

Chapter 4 addresses the effects of feeding and oral secretions of two caterpillar species on the gene transcription of several defense genes in Lima bean. Moreover, it explores the effects on defense gene induction by a subsequently arriving herbivore.

Chapter 5 investigates how defense induction by a heterospecific non-prey herbivore affects indirect defense against *T. urticae* that were inoculated subsequently.

Chapter 6 investigates the effect of conspecific herbivores on plant memory formation in terms of indirect defense against *T. urticae*.

The last chapter of this thesis, Chapter 7, summarizes and discusses the main results of this thesis.

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Chapter 2
Defense gene induction in Lima
bean in
response to phytohormone
application and
spider-mite feeding

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Abstract

Phytohormones play an important role in plant defense by regulating gene transcription of defense-related genes. Exogenous application of phytohormones can be used to simulate herbivory and induce gene transcription. Here we studied the response of three defense-related genes, *Phaseolus lunatus* lipoxygenase (*PILOX*), *Phaseolus lunatus* β -ocimene synthase (*PIOS*) and *Phaseolus lunatus* Pathogenesis-related protein 4 (*PIPR-4*) to different doses of phytohormones and different densities of the herbivore *Tetranychus urticae*. Transcript levels of the three defense genes of Lima bean plants were quantified at 48 hours after initiation of treatment. They differently correlated to phytohormone dose and herbivore density. The jasmonic acid (JA)-responsive gene *PIOS* responds to JA and salicylic acid (SA) and reflected the antagonistic interactions between the JA and SA phytohormone signaling pathways. Moreover, *PIOS* transcript positively correlated to herbivory by to JA-inducing *T. urticae* mites. The JA-responsive gene *PILOX*, did not show a correlation to phytohormone doses or herbivore densities at the time point investigated here. The *PIPR-4*, previously reported to respond to methyl salicylate-responsive gene, did not respond to treatment with SA.

Introduction

Plants rely on a sophisticated network of defense signaling pathways and their fine-tuned regulation to generate an effective defense response against attacking herbivorous arthropods. Defense signal-transduction pathways are the central part of so-called induced plant defenses. This type of plant defense is highly plastic and allows plants to vary investment in defense in comparison to the static constitutive defenses (Schoonhoven *et al.*, 2005). The signaling pathways ultimately result in the activation of transcription of genes involved in plant defense, which in turn results in production of secondary plant metabolites that mediate plant defense (Stam *et al.*, 2014).

Depending on the type of herbivore that attacks a plant, different signal-transduction pathways can be induced. The main signaling pathways are the jasmonic acid (JA) pathway, also known as octadecanoid pathway, and the salicylic acid (SA) pathway, also known as shikimate pathway (Pieterse *et al.*, 2012). Moreover, the ethylene pathway plays an important role in potentiating the JA pathway. Generally, biting-chewing herbivores and certain cell content-feeders induce the JA pathway, whereas phloem-feeding herbivores activate the SA pathway (McConn *et al.*, 1997; Li *et al.*, 2002; Kempema *et al.*, 2007; Pieterse *et al.*, 2012). The two pathways act antagonistically and thus induction of one signaling pathway may interfere with the induction of defense mechanisms regulated by the other pathway (Thaler *et al.*, 2012). The application of phytohormones can be used to effectively mimic herbivory in initiating signal transduction (e.g. Boland *et al.*, 1995; Dicke *et al.*, 1999; Gols *et al.*, 1999; Ament *et al.*, 2004; Lou *et al.*, 2005; Ozawa *et al.*, 2008; Zhang *et al.*, 2009; Wei *et al.*, 2014).

In this study we investigated the effects of phytohormone application and actual herbivory by *T. urticae* on transcript levels of three genes involved in plant defense. The *Phaseolus lunatus* lipooxygenase (*PILOX*) gene is involved in the JA signaling pathway. Transcription of the *Phaseolus lunatus* β -ocimene synthase (*PIOS*) gene is induced through the JA pathway and is involved in the production of the monoterpene plant volatile (*E*)- β -ocimene (Ament *et al.*, 2004; Arimura *et al.*, 2008). The latter compound serves an important function in plant indirect defense, by attracting natural enemies such as the predatory mite *Phytoseiulus persimilis* to Lima bean plants infested with the spider mite *Tetranychus urticae* (Dicke *et al.*, 1990). Transcription of both genes, *PILOX* and *PIOS*, is expected to be induced by increased JA levels caused by exogenous application of the phytohormone itself, and to be suppressed by exogenous application of the antagonistic phytohormone SA. Because *T. urticae* mites have also been shown to induce JA in Lima bean plants, spider mite feeding is expected to exert an influence on JA-responsive genes by naturally increasing endogenous levels via feeding damage (Li *et al.*, 2002). In contrast, chitinases, such as the pathogenesis-related (PR) protein 4, are commonly induced in plant tissues in response to SA-related signal transduction (Ward *et al.*, 1991; Arimura *et al.*, 2000). Consequently, we expected *Phaseolus lunatus* PR-protein 4 to respond to SA treatment.

Materials and Methods

Plants and mites

Lima bean plants (*Phaseolus lunatus* L., cv. Wonderbush, De Bruyn Seed Company, Michigan, USA) were cultivated in a greenhouse compartment at 23 ± 2 °C, 60 ± 10 % relative humidity (RH) and a 16 : 8 h light : dark (L : D) photoregime. Plants were grown in 11 x 11 cm plastic pots. After 12 - 14 days plants with two expanded primary leaves were transferred to a climate chamber and incubated at 25 ± 1 °C, 60 ± 10 % R.H. and 16 : 8 h L : D. A colony of two-spotted spider mites, *Tetranychus urticae* Koch (Acari: Tetranychidae), was maintained on Lima bean plants in a greenhouse compartment at 25 ± 5 °C, 50 – 70 % R.H., 16 : 8 h L : D. Adult female spider mites that were used in the experiments were selected randomly from the colony.

Plant treatments

Phytohormone treatment

To determine the effect of phytohormones on gene transcription, a dose-response experiment was performed. Primary leaves of Lima bean plants were sprayed with 1 ml per leaf of JA or SA solutions at different concentrations (see below) in water or with 1 ml of water as a control. JA and SA were purchased from Sigma-Aldrich. The plants were left to dry for 30 - 60 min. After phytohormone or control treatment, plants were transferred to a climate chamber and incubated in cages (metal frame 90 x 90 x 60 cm, walls of polyethylene sheet) separated by treatment at 23 ± 2 °C, 60 ± 10 % RH and 16L : 8D. Each cage contained 6-9 plants per treatment per experiment. The building's vacuum system was connected to the top of each cage with a suction of approximately 7 L/min. The four treatments for the JA dose response curve were: i) water, ii) 0.01 mM JA, iii) 0.1 mM JA, and iv) 1 mM JA. The five treatments for the SA dose response curve were: i) water, ii) 0.001 mM SA, iii) 0.01 mM SA, iv) 0.1 mM SA, and v) 1 mM SA. Plants were incubated for 48 h after their respective treatment.

Spider mite density

To investigate the effects of spider-mite density on gene transcription, adult female mites were evenly distributed over the two primary leaves of plants from the respective treatments using a fine paint brush. Treatments consisted of i) no mites (control), ii) eight *T. urticae* mites, iii) 20 *T. urticae* mites, and iv) 50 *T. urticae* mites. After 48 h of incubation, the mites and their products (webbing, eggs) were removed using a fine paint brush.

RNA extraction and cDNA synthesis

After 48 h of incubation, plant material was collected and processed as previously described in Menzel *et al.* (2014). In short, 2-3 biological replicates were collected for each treatment per experiment. Each biological replicate consisted of plant leaf material pooled from primary leaves of three plants. Experiments were repeated up to two times.

Quantitative RT-PCR

A real-time quantitative RT-PCR was used to quantify gene transcript levels of *P. lunatus* β -ocimene synthase (*PIOS*; GenBank accession EU194553), *acidic pathogenesis-related protein 4* (*PIPR-4*), and the two reference genes *P. lunatus Actin1* (*PIACT1*; GenBank accession DQ159907) and *P. lunatus Nuclear matrix protein 1* (*PINMP1*; GenBank accession AF289260.1). Quantitative RT-PCR was performed in a Rotor-Gene 6000 machine (Corbett Research) with a 72-well rotor; for a detailed description see Menzel *et al.* (2014). Relative gene transcripts for assessing the dose-response curve with JA and mite density were calculated with two reference genes, while relative gene transcripts for SA dose response were calculated only with *PIACT1*, simply omitting the calculation of a geometric average. *PIOS* primers were F-*PIOS* 5'-TGCATGGGTCTCAGTCTCTG-3' and R-*PIOS* 5'-TGCTGCTTCCCCTCTCTCTA-3', *PIPR-4* were F-*PIPR-4* 5'-ACGCTTTCCTCAGTGCTCTC-3' and R-*PIPR-4* 5'-TCCTCGTCGTCGAGTAATCCTT-3', *PIACT1* primers were F-*PIACT1* 5'-CCAAGGCTAACCGTGAAAAG-3' and R-*PIACT1* 5'-AGCCAGATCAAGACGAAGGA-3', and *PINMP1* primers were F-*PINMP1* 5'-CCGGAATGGAGTGTTGACGAGCA-3' and R-*PINMP1* 5'-CCAGCTCAGAAACATCTGGCAATGG-3'.

Statistical analysis

Log transformation was applied to data from gene transcription experiments in order to meet assumptions of normality and homogeneity of variances. Correlations between gene transcript levels and phytohormone doses or spider mite density, were analyzed using Pearson's correlation tests in the statistical software SPSS version 19 (SPSS Inc., Chicago, IL, USA). Data that violated assumptions on normality and equal variance after log transformation were analyzed by Spearman's rank correlation test.

Results

In response to exogenous application of different doses of JA, a positive correlation with *PIOS* transcript levels was found (Fig 1A, Spearman's correlation, $r = 0.76$, $P < 0.05$). Furthermore, transcript levels of *PIOS* showed a negative correlation with increasing SA doses (Fig 1B, Pearson's correlation, $r = -0.81$, $P < 0.001$). The density of *T. urticae* mites was also positively correlated with *PIOS* transcript levels (Fig. 1C, Spearman's, $r = 0.74$, $P < 0.001$).

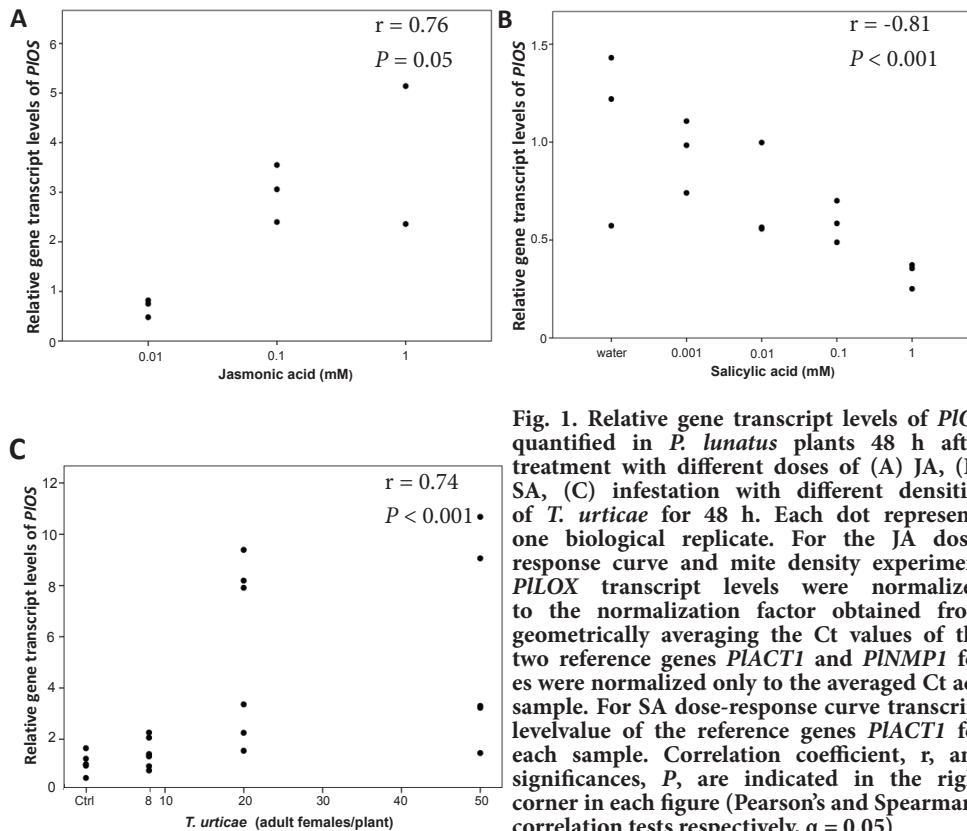


Fig. 1. Relative gene transcript levels of *PIOS* quantified in *P. lunatus* plants 48 h after treatment with different doses of (A) JA, (B) SA, (C) infestation with different densities of *T. urticae* for 48 h. Each dot represents one biological replicate. For the JA dose-response curve and mite density experiment *PILOS* transcript levels were normalized to the normalization factor obtained from geometrically averaging the Ct values of the two reference genes *PIACT1* and *PIINMP1* for each sample. For SA dose-response curve transcript level value of the reference genes *PIACT1* for each sample. Correlation coefficient, r , and significances, P , are indicated in the right corner in each figure (Pearson's and Spearman's correlation tests respectively, $\alpha = 0.05$).

Lipoxygenase is a central enzyme in the wound-induced biosynthesis of JA via the octadecanoid pathway (Bell *et al.*, 1995). However, transcript levels of *PILOS* showed no correlation with the dose of the phytohormones JA or SA at 48 h after treatment (Fig 2 A, B, Pearson's correlation, both $P > 0.05$). *PILOS* transcript levels also did not correlate with the density of *T. urticae* mites (Fig. 2 C, Spearman's, $P > 0.05$).

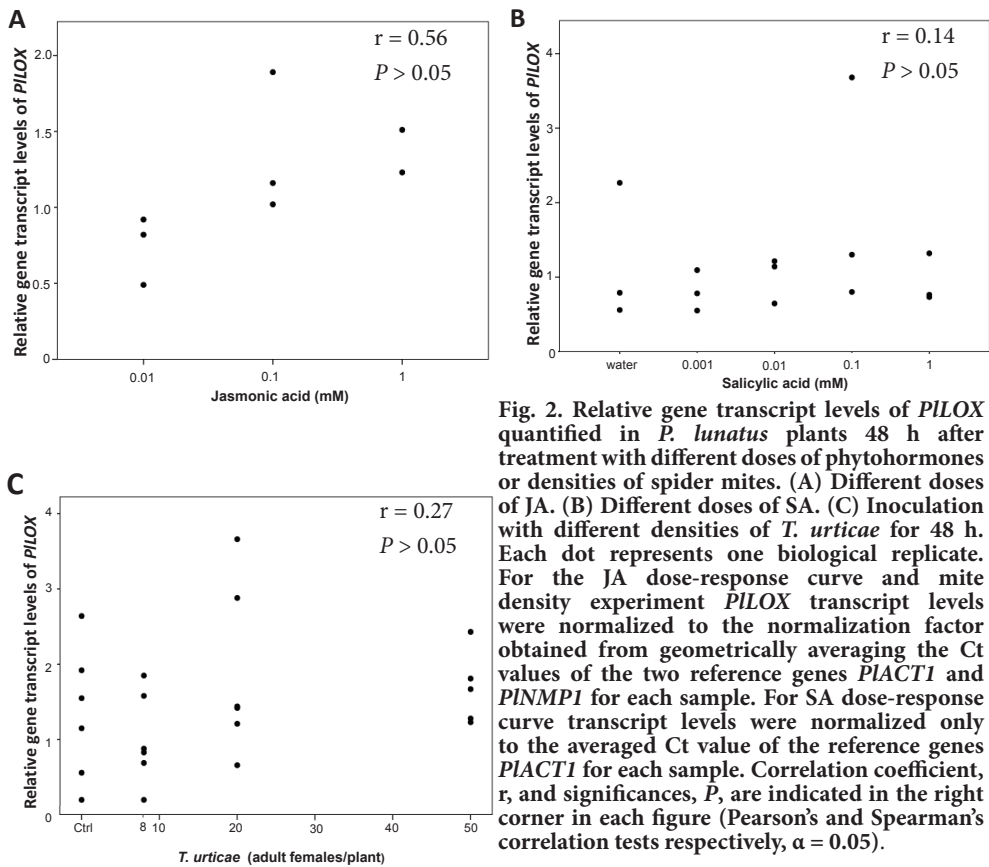


Fig. 2. Relative gene transcript levels of *PILOX* quantified in *P. lunatus* plants 48 h after treatment with different doses of phytohormones or densities of spider mites. (A) Different doses of JA. (B) Different doses of SA. (C) Inoculation with different densities of *T. urticae* for 48 h. Each dot represents one biological replicate. For the JA dose-response curve and mite density experiment *PILOX* transcript levels were normalized to the normalization factor obtained from geometrically averaging the Ct values of the two reference genes *PIACT1* and *PINMP1* for each sample. For SA dose-response curve transcript levels were normalized only to the averaged Ct value of the reference genes *PIACT1* for each sample. Correlation coefficient, r , and significances, P , are indicated in the right corner in each figure (Pearson's and Spearman's correlation tests respectively, $\alpha = 0.05$).

Transcript levels of *PIPR-4* did not show a correlation with the dose of the phytohormone SA (Fig. 3, Pearson's correlation, $r = -0.27$, $P > 0.05$).

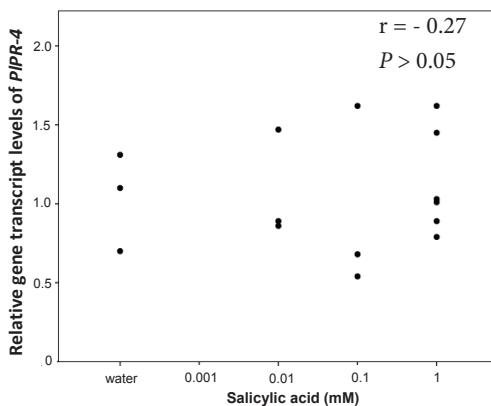


Fig. 3. Relative gene transcript levels of *PIPR-4* quantified in *P. lunatus* plants 48 h after treatment with different doses of SA. Each dot represents one biological replicate. *PIPR-4* transcript levels were normalized to the normalization factor obtained from geometrically averaging the Ct values of the two reference genes *PIACT1* and *PINMP1* for each sample.

For the JA dose-response curve and mite density experiment, *PIOS* transcript levels were normalized to the normalization factor obtained from geometrically averaging the Ct values of the two reference genes *PIACT1* and *PINMP1* for each sample. For the SA dose-response curve transcript levels were normalized only to the averaged Ct value of the reference genes *PIACT1* for each sample. Correlation coefficient, r , and significances, P , are indicated in the right corner in each figure (Pearson's and Spearman's correlation tests respectively, $\alpha = 0.05$).

Discussion

Phytohormonal signaling pathways are vital for the regulation of induced plant defense. Here, we investigated the effect of phytohormone application and herbivory on gene transcript levels of three defense-related genes, i.e. *PIOS*, *PILOX*, and *PIPR-4*, in Lima bean plants. The results show that at 48 h after initiation of treatment transcript levels of one of the three defense genes correlated with phytohormone doses and herbivore density. A positive correlation was found between exogenous JA doses and density of *T. urticae* mites and *PIOS* transcript levels. This is in accordance with Arimura *et al.* (2008) who demonstrated for Lima bean that while *PIOS* is only active during the light period, it follows the pattern of endogenous JA levels. In our study, also the infestation level of *T. urticae* mites correlated positively with *PIOS* transcript levels. *Tetranychus urticae* are known to induce the octadecanoid pathway and an intact JA-related signaling is required to induce direct and indirect defense against these herbivores (Ozawa *et al.*, 2000; Li *et al.*, 2002; Ament *et al.*, 2004). Moreover, we found a negative correlation between SA application and *PIOS* transcript levels. It has been shown that herbivores that induce SA, such as whiteflies, decrease *PIOS* transcript levels and thus interfere with JA-induced defenses (Zhang *et al.*, 2009). The down-regulation of *PIOS* transcription is thus probably caused by the down-regulation of endogenous JA levels because of the negative cross-talk between the JA and the SA signaling pathways (Koornneef and Pieterse, 2008). Nevertheless, the transcript levels of a gene located in the octadecanoid pathway, *PILOX*, did not show correlation between phytohormone doses or herbivore density at the time point investigated. The LOX gene catalyzes an early step of the JA signaling pathway in response to wounding (Bell *et al.*, 1995). Zhang *et al.* (2009) found a negative effect of the SA-inducing herbivore, *Bemisia tabaci*, on *PILOX* when plants were co-infested with *T. urticae* and whiteflies after 12 h. Moreover, Arimura *et al.* (2000) found an induction of *PILOX* 24 h after exogenous application of JA. The apparent discrepancy might be explained by the different sampling time points compared to the current experiments. LOX is known to be induced early in response to herbivory and LOX is upstream of OS which may mean that while at 48 h since treatment *PIOS* induction was demonstrated, the peak of *PILOX* transcription has already passed. Indeed, 12 h after the initiation of spider mite infestation JA is induced in Lima bean, suggesting that *PILOX* expression is upregulated earlier than 12 h since treatment (Zhang *et al.* 2009).

Pathogenesis-related genes are commonly associated with SA-mediated defense responses and exogenous application of SA usually activates the expression of these defense genes (Yalpani *et al.*, 1991). In fact, Arimura *et al.* (2000) found that an incubation of Lima bean plants with the methylated form of SA, methyl salicylate, results in the induction of acidic *PIPR-4* in Lima bean plants 24 h after application. However, in our experiment no correlation between transcript levels of *PIPR-4* and SA was found at the investigated time point.

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Chapter 3

**Synergism in the effect of prior
jasmonic acid application on
herbivore-induced volatile emission
by Lima bean plants: transcription
of a monoterpene synthase gene and
volatile emission**

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Abstract

Jasmonic acid (JA) plays a central role in induced plant defense e.g. by regulating the biosynthesis of herbivore-induced plant volatiles which mediate the attraction of natural enemies of herbivores. Moreover, exogenous application of JA can be used to elicit plant defense responses similar to those induced by biting-chewing herbivores and mites that pierce cells and consume their contents. In the present study, we used Lima bean (*Phaseolus lunatus*) plants to explore how application of a low dose of JA followed by minor herbivory by spider mites (*Tetranychus urticae*) affects transcript levels of *P. lunatus* (*E*)- β -ocimene synthase (*PIOS*), emission of (*E*)- β -ocimene and 9 other plant volatiles commonly associated with herbivory. Furthermore, we investigated the plant's phytohormonal response. Application of a low dose of JA increased *PIOS* transcript levels in a synergistic manner when followed by minor herbivory for both simultaneous and sequential infestation. Emission of (*E*)- β -ocimene was also increased, and only JA, but not SA, levels were affected by treatments. Projection to Latent Structures-Discriminant Analysis (PLS-DA) of other volatiles showed overlap between treatments. Thus, a low-dose JA application results in a synergistic effect on gene transcription and an increased emission of a volatile compound involved in indirect defense after herbivore infestation.

Introduction

Plants possess a whole arsenal of mechanisms to resist attacks by pathogens and herbivorous arthropods. The basis of induced plant resistance against insect herbivory consists of a complex network of phytohormonal signaling. A general component of the response to chewing herbivores and foliar wounding is elicitation of the jasmonic acid (JA) signaling pathway in which the phytohormone JA plays a central role (McConn *et al.*, 1997; Kessler and Baldwin, 2002). In contrast, piercing-sucking insects and biotrophic pathogens commonly induce the salicylic acid (SA) signaling pathway, which antagonizes the JA pathway (Kempema *et al.*, 2007; Thaler *et al.*, 2012). Both pathways regulate large-scale changes in defense-related parts of the plant transcriptome, proteome, and metabolome, which underlie plant direct and indirect resistance mechanisms (Kessler and Baldwin, 2002; Pieterse and Dicke, 2007).

Biosynthesis of JA is initiated by the perception of herbivore- and damage-associated molecular patterns (HAMPs and DAMPs, respectively), which accompany herbivore attack and mechanical damage of plant tissue (Mithöfer & Boland 2008). The synthesis and accumulation of the JA-isoleucine conjugate, JA-Ile, generally causes a derepression of relevant transcription factors and defense-related genes in the plant (Boter *et al.*, 2004; Lorenzo *et al.*, 2004; Chini *et al.*, 2007; Thines *et al.*, 2007). Activation of these JA-responsive genes then leads to the production of metabolites involved in plant resistance. Local activation of JA signaling also results in the production of signaling molecules that can spread systemically through the plant and induce JA responses in distant organs, where they provide protection against imminent attackers (Ryan, 2000; Koo *et al.*, 2009). While many processes within the JA pathway have been widely studied, the identity of specific gene products and metabolites that account for JA-mediated resistance are still unknown in most non-model plant species for which genomic sequence information is not yet available.

The role of the JA pathway in the regulation of induced plant volatile synthesis has been well studied. Early and late intermediates of the JA pathway as well as the final product, JA, induce synthesis of volatiles, which serve an important function in plant interactions with arthropods (Dicke *et al.*, 1999; Koch *et al.*, 1999; Bruinsma *et al.*, 2009a; Snoeren *et al.*, 2009; Bruinsma *et al.*, 2010). Volatile compounds that are synthesized *de novo* or in increased amounts by attacked plants are called herbivore-induced plant volatiles (HIPVs). These compounds are particularly involved in mediating tritrophic interactions, in which natural enemies of herbivores use plant volatiles as cues to locate their herbivorous host or prey (Mumm and Dicke, 2010). While many of these compounds have been identified, another level of complexity is posed by the fact that the exact expression of the defense response by a plant is often modulated by the ecological context. Timing, intensity, and other characteristics of the defense response are influenced by factors such as the specific nature of the attacker (Takabayashi *et al.*, 1995; De Moraes *et al.*, 1998; Stout *et al.*, 1998; De Vos *et al.*, 2005), ontogenetic stage of the attacked plant (Hare, 2010) and plant tissue (Wentzell and Kliebenstein, 2008), population density of plants and density of attackers (Gols *et al.*, 2003; Wentzell and Kliebenstein, 2008; Kegge *et al.*, 2013). Moreover, plant defenses are further modulated by the simultaneous presence of multiple herbivores and pathogens on the same plant (Moayeri *et al.*, 2007; Dicke *et al.*, 2009),

as well as previous infestations (Stout *et al.*, 1998; Jung *et al.*, 2009; Ponzio *et al.*, 2013).

Exogenous application of key phytohormones in defense signaling pathways can be used to elicit plant defense responses similar to those induced by arthropod herbivores or pathogens (Dicke *et al.*, 1999; Gols *et al.*, 1999; Koornneef *et al.*, 2008). Treatment of plants with JA, or its volatile derivative methyl jasmonate (MeJA), has been shown to confer broad resistance against plant attackers such as nematodes (Cooper *et al.*, 2005), biting-chewing insects (Omer *et al.*, 2000; Tierranegra-García *et al.*, 2011), and necrotrophic pathogens (Brader *et al.*, 2001; Yamada *et al.*, 2012). Even plants grown from seeds previously exposed to JA, have been found to be more resistant to herbivory (Worrall *et al.*, 2012). Observed JA-mediated resistance is attributed to enhanced induction of direct resistance mechanisms, such as accumulation of plant toxins or proteinase inhibitors, or indirect resistance mechanisms, that promote the effectiveness of natural enemies of plant attackers. Generally, application of JA induces volatile blends that are similar to those induced by herbivory (Dicke *et al.*, 1999; Gols *et al.*, 1999; Kessler and Baldwin, 2001). These volatile blends consist of compounds that can be exploited by natural enemies as cues to locate their herbivorous prey or host. Several studies have investigated the effect of phytohormonal induction on indirect resistance (e.g. Dicke and Vet, 1999; Gols *et al.*, 1999; Ozawa *et al.*, 2000; Bruinsma *et al.*, 2008; Bruinsma *et al.*, 2009b). Phytohormone application allows for manipulation of defined steps in signal-transduction pathways and to induce plants in a dose-controlled manner without removal of plant tissue.

In the present study, we have explored how a low JA-dose affects Lima bean indirect defense against the generalist herbivorous mite *Tetranychus urticae*. JA is a key regulator of the induction of volatiles emitted in response to *T. urticae* infestation such as (*E*)- β -ocimene (Dicke *et al.*, 1999; Ament *et al.*, 2004). The monoterpene (*E*)- β -ocimene is an HIPV released in response to herbivory by a range of plant species including cucumber, apple, Lima bean, cotton, corn, and tobacco (Paré and Tumlinson, 1999). Moreover, (*E*)- β -ocimene is one of the five principle compounds that mediate the attraction of the specialist predator *Phytoseiulus persimilis* to *T. urticae*-infested plants (Dicke *et al.*, 1990; De Boer and Dicke, 2004).

Gols *et al.* (2003) found that treatment of Lima bean plants with a low dose of JA, which in itself did not result in attraction of the predatory mite *P. persimilis*, resulted in an enhanced attraction of *P. persimilis* in response to herbivory by a low density of spider mites. Enhanced predator attraction was still found when a time lapse of 7 days was introduced between the treatment with JA and the infestation of spider mites. Here, we investigated the underlying mechanism. We hypothesized that exogenous application of a low dose of JA to Lima bean would induce JA-responsive gene transcription and subsequent terpene emissions with a priming or additive effect when followed by minor herbivory. We have focused on the transcription of the *Phaseolus lunatus* β -ocimene synthase (*PIOS*) gene. *PIOS* codes for the enzyme *ocimene synthase* that mediates the rate-limiting step in the biosynthesis of (*E*)- β -ocimene (Ament *et al.*, 2004; Arimura *et al.*, 2004).

Materials and Methods

Plants and mites

Lima bean plants (*Phaseolus lunatus* L., cv Wonderbush) were sown and grown in a greenhouse compartment at 23 ± 2 °C with 60 ± 10 % R.H., and a photoperiod of 16L : 8D. Plants having two fully expanded primary leaves were used for experiments at 12 - 15 days after sowing. Two-spotted spider mites, *Tetranychus urticae* Koch (Acari: Tetranychidae), were reared on Lima bean plants in a different greenhouse compartment under the same conditions as the Lima bean plants. Only adult female mites were used for experiments.

Treatments

Combined effects of JA and simultaneous or sequential spider-mite infestation on PLOS transcription

Primary leaves of Lima bean plants were sprayed with 1 ml per leaf of 0.1 mM JA solution (Sigma-Aldrich) in water or with 1 ml of water as a control. The plants were left to dry for 30 - 60 min. After phytohormone or control treatment, plants were transferred to a climate chamber and incubated separated by treatment in cages (metal frame 90 x 90 x 60 cm, walls of polyethylene sheet) at 23 ± 2 °C, 60 ± 10 % RH and 16L : 8D. Each cage contained 16 plants per treatment for gene transcription and phytohormone analysis or four plants per treatment for volatile trapping experiments. The building's vacuum system was connected to the top of each cage with a suction of approximately 7 L/min to avoid interactions through volatiles between plants of different treatments.

The four treatments were: i) water, ii) water and mites, iii) JA, and iv) JA and mites. For simultaneous infestations, spider mites were applied after plants sprayed with JA solutions were dry. Four adult female mites were evenly distributed over the two primary leaves of plants from the respective treatments using a fine paint brush. Mites were randomly selected from the spider-mite culture. After two days of incubation, the mites and their products (webbing, eggs) were removed using a fine paint brush.

In subsequent experiments with sequential infestation, mites were inoculated seven days after JA treatment and transferred to cages as described above. Two days before mite application, lanolin paste was applied around the petioles of both primary leaves of each plant to confine the mites to the leaves. After a seven day incubation period, leaf material from plants of treatments i) water and iii) JA was collected. The two other treatments, i.e. ii) water and mites, and iv) JA and mites, received the mite treatment (four adult females per plant) and were incubated for another two days, after which leaf material was collected.

RNA extraction and cDNA synthesis

Leaf material was collected by excising four leaf discs at 12:00 - 13:00h from a primary leaf using a cork borer (diameter 2 cm), and the leaf discs obtained from three plants were pooled to give one biological replicate. Upon collection, samples were immediately shock-frozen in liquid nitrogen and stored at -80 °C until processing. The leaf material was homogenized without thawing using a mortar and pestle. Total RNA was extracted and purified using the Qiagen RNeasy Plant Mini kit with integrated DNase treatment, following the manufacturer's instructions. Absence of genomic DNA contamination and RNA quality were assessed using Agilent 2100 Bioanalyzer with the RNA 6000 Nano Labchip® kit (all from Agilent Technologies). RNA was quantified with a NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Only RNA samples with 260 / 280 wavelength ratio > 2 and a RIN value > 7 were used for cDNA synthesis. cDNA was generated from total RNA by using the Bio-Rad iScript cDNA synthesis kit (Bio-Rad), following the manufacturer's instructions.

Quantitative RT-PCR

Transcript levels of *P. lunatus* β -ocimene synthase (*PIOS*; GenBank accession EU194553) and the two reference genes *P. lunatus* *Actin1* (*PIACT1*; GenBank accession DQ159907) and *P. lunatus* *Nuclear matrix protein 1* (*PINMP1*; GenBank accession AF289260.1) were quantified by performing a real-time quantitative RT-PCR in a Rotor-Gene 6000 machine (Corbett Research) with a 72-well rotor. Reactions were performed in a final volume of 25 μ l, that included 12 μ l iQTM SYBR® Green Supermix (Bio-Rad), 1 μ l forward primer (4 μ M) and reverse primer (4 μ M) pairs (final primer concentration: 160 nM), and 5 μ l cDNA (4 ng/ μ l) first strand template. The PCR program for *PIOS* and the reference gene *PIACT1* was the same as described by Zheng *et al.* (2007). The *PIOS* primers were F-*PIOS*5'-TGCATGGGTCTCAGTCTCTG-3' and R-*PIOS*5'-TGCTGCTTCCCCTCTCTCTA-3' with a predicted product length of 189 bp. *PIACT1* primers were F-*PIACT1* 5'-CCAAGGCTAACCGTGAAAAG-3' and R-*PIACT1* 5'-AGCCAGATCAAGACGAAGGA-3' with predicted product length of 208 bp. The second reference gene, *PINMP1*, was designed with the Geneious software version 4.8.3 under default parameters except that the annealing temperature was set to 56 °C. Predicted product length of the *PINMP1* primers F-*PINMP1* 5'-CCGGAATGGAGTGTTGACGAGCA-3' and R-*PINMP1* 5'-CCAGCTCAGAAACATCTGGCAATGG-3' was 157 bp. The PCR program for *PINMP1* was adapted from Zheng *et al.* (2007), whereby the extension time was increased from 45 s to 48 s. Specificity of amplicons was verified for each primer pair by melt-curve analysis to assure absence of nonspecific products as well as primer-dimer formation. Relative quantification of *PIOS* transcription was calculated with the 2^{(-Delta Delta C(T))} method (Livak and Schmittgen, 2001), using a normalization factor (Vandesompele *et al.*, 2002). The normalization factor was calculated by geometrically averaging the threshold cycle (Ct) values from the two reference genes *PIACT1* and *PINMP1* ($M < 0.03$, GeNorm). Subtraction of the normalization factor from *PIOS* Ct values normalizes for differences in cDNA synthesis.

Phytohormone quantification

Quantification of JA and SA levels in samples used for gene transcription analysis followed the protocol of Schulze *et al.* (2006). Samples were analyzed on a Finnigan ITQ Instrument (Thermo Electron, Bremen, Germany) running in a CI-negative ion mode.

Dynamic headspace collection of plant volatiles

Collection of plant volatiles was carried out in 20-L glass jars sealed with a viton-lined glass lid with an inlet and outlet. Compressed air was filtered by passing through charcoal before entering the glass jar containing the plant. Volatiles were collected by sucking air out of the glass jar at a constant rate of 200 ml/min through a stainless steel tube filled with 200 mg Tenax TA (Markes, Llantrisant, UK) for 2 h. Before sampling, empty glass jars were purged with compressed air for 1 h. Pots in which the plants had grown were removed, roots and soil were carefully wrapped in aluminum foil, then the plant was placed in a glass jar. The glass jars containing the plants were flushed for an additional 30 min before connecting stainless steel tubes filled with Tenax TA. Plant volatiles were collected from seven replicates of each treatment i) water, ii) water and mites, iii) JA, and iv) JA and mites. Fresh weight of above-ground plant tissue was determined immediately after volatile collection using an analytical balance (NewClassic ML, Mettler Toledo, Switzerland).

Analysis of plant volatiles

Thermo Trace GC Ultra coupled with Thermo Trace DSQ quadrupole mass spectrometer (Thermo Fisher Scientific, Waltham, USA) was used for separation and detection of plant volatiles. Prior to release of the volatiles, each sample was spiked with 10 ng/ μ l of 1-bromodecane as internal standard (I.S.) and dry-purged under a stream of nitrogen (50 ml/min) for 10 min at ambient temperature in order to remove moisture and the organic solvent methanol used to prepare the I.S. The collected volatiles and I.S. were released from the Tenax TA using the Ultra 50 : 50 thermodesorption unit (Markes) at 250 °C for 10 min under helium flow of 20 ml/min, while re-collecting the volatiles in a thermally cooled universal solvent trap at 10 °C using Unity (Markes). Once the desorption process was completed, volatile compounds were released from the cold trap by ballistic heating at 40 °C/s to 280 °C. The temperature was kept at 280 °C for 10 min, while the volatiles were transferred to a ZB-5MSi analytical column [30 m x 0.25 mm I.D. x 1.00 μ m F.T. (Phenomenex, Torrance, CA, USA)], in a splitless mode for further separation. The GC oven temperature was initially held at 40 °C for 2 min and was raised at 10 °C/min to a final temperature of 280 °C, where it was kept for 4 min under a helium flow of 1 ml/min in a constant flow mode. The DSQ mass spectrometer (MS) was operated in a scan mode with a mass range of 35 – 350 amu at 5.38 scans/s and spectra were recorded in electron impact ionization (EI) mode at 70 eV. MS transfer line and ion source were set at 275 and 250 °C, respectively. Compound identification was based on retention time of authentic standards and comparison of mass spectra with those in the NIST 2005 and Wageningen Mass Spectral Database of Natural Products MS libraries. Experimentally calculated linear retention indices (LRI) were also used as additional measure to confirm the identity of compounds.

Standards of (*E*)-2-hexenal, (*Z*)-3-hexen-1-ol, (*Z*)-3-hexen-1-ol acetate, (*E*)- β -ocimene, linalool, methyl salicylate (MeSA), indole, caryophyllene as well as the internal standard (I.S.) 1-bromodecane, a series of alkane mixtures (C8 – C20) and the solvent methanol (GC grade) were obtained from Sigma-Aldrich (Saint Louis, MO, USA). Additional standards (*E*)-4,8-dimethylnona-1,3,7-triene [(*E*)-DMNT] and (*E,E*)-4,8,12-trimethyltrideca-1,3,7,11-tetraene [(*E,E*)-TMTT] were synthesized at the Max Planck Institute of Chemical Ecology (Jena, Germany) following the procedure by Boland and Gabler (1989). For quantification, calibration lines were constructed for each compound using seven data points at different concentrations (two replicates of each data point) and was carried out using a single (target) ion, in selected ion monitoring (SIM) mode.

Statistical analysis

Univariate data, i.e. gene transcription and plant volatile data were log-transformed to meet the test assumptions of normality and homogeneity of variances. Phytohormone data were analyzed without transformation. Analyses were performed using one-way ANOVA followed by Fisher's least significant difference (LSD) post-hoc tests for pairwise comparisons between treatments in the statistical software SPSS version 19 (SPSS Inc., Chicago, IL, USA). If assumptions on normality and equal variance were violated, Kruskal-Wallis tests followed by Mann-Whitney U tests with a Bonferroni correction as post-hoc tests were used. Assumption of synergism was tested by subtraction of baseline levels of both single treatments and subsequent summation. If the resulting value was outside the 95 % confidence interval of the mean from a combination treatment, the interaction between the single treatments was considered significantly different.

Effects of treatments, time of trapping and the interaction on (*E*)- β -ocimene emission were analyzed by general linear model (GLM) with LSD post-hoc tests. Evaluation of differences between treatments of morning trapping and afternoon trapping were done by an one-way ANOVA followed by Fisher's least significant difference (LSD) post-hoc tests for pairwise comparisons.

The multivariate data analysis of plant volatiles corrected by fresh weight using Projection to Latent Structures-Discriminant Analysis (PLS-DA) was performed to test for differences in volatile profiles among different treatments. The analysis was carried out using the software SIMCA P+ version 12 (Umetrics, Umea, Sweden). Data were log-transformed and univariate-scaled prior to PLS-DA analysis.

Results

Transcriptional changes in *PIOS* levels in response to JA and spider-mite treatment

Transcript levels of *PIOS* in response to the treatments, i.e. i) water (control), ii) 0.1 mM JA, iii) four *T. urticae*, and the combined treatment iv) 0.1 mM JA with simultaneous inoculation of four *T. urticae* showed significant differences (Fig. 1A).

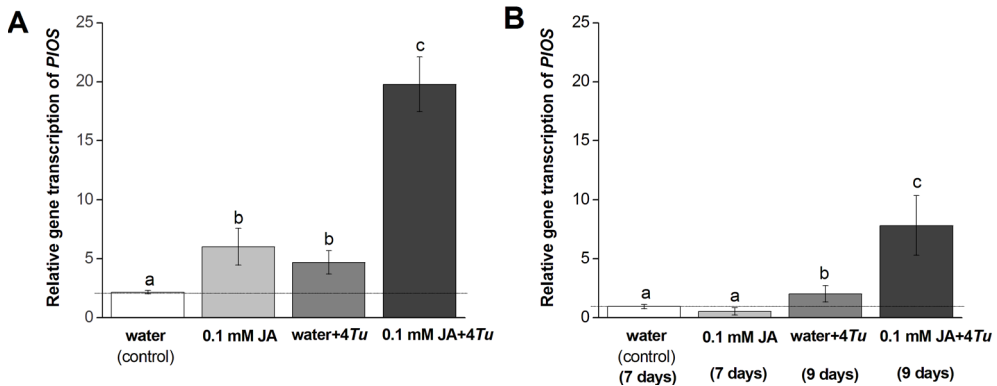


Fig. 1. Relative gene transcript levels of *PIOS* quantified in *P. lunatus* plants treated with i) water (control), ii) 0.1 mM JA, iii) four *T. urticae* (water + 4*Tu*), or iv) 0.1 mM JA with four *T. urticae* mites (0.1 mM JA + 4*Tu*). (A) Inoculation of four adult female *T. urticae* on plants was done immediately following JA-treatment and mites had since been feeding for 48 h, and (B) inoculation of four adult female *T. urticae* was done seven days after incubation with water or 0.1 mM JA and mites had since been feeding for 48 h. Values are the mean (\pm SE) of three to four biological replicates, different letters above bars indicate significant differences in transcript levels between treatments (ANOVA followed by Fisher's LSD test, $\alpha = 0.05$). *PIOS* transcript levels were normalized to the normalization factor obtained from geometrically averaging the Ct values of the two reference genes *PIACT1* and *PINMP1* for each sample. Baseline represents transcript level in control plants.

Plants treated with 0.1 mM JA or four *T. urticae* alone showed higher ($P < 0.05$ for both comparisons) *PIOS* transcript levels after 48 h compared to control plants, but did not differ from each other. Plants treated with the combination of 0.1 mM JA and four simultaneously inoculated *T. urticae* also showed higher ($P < 0.01$) *PIOS* levels after 48 h compared to control and the single treatment with JA or mites. The combination treatment resulted in a *PIOS* transcript level that is twice the level that would be obtained if the effects of JA and four *T. urticae* were additive, revealing a synergistic effect of the two treatments on *PIOS* transcript levels.

Significant differences between treatments were also found in the second experiment in which inoculation of *T. urticae* was done seven days after the application of 0.1 mM JA or water ($P < 0.05$; Fig. 1B). *PIOS* transcript levels in plants treated with 0.1 mM JA were not significantly different from control plants after seven days of incubation. When four *T. urticae* were inoculated on water-treated plants at this time point and incubated for another two days, the *PIOS* transcript level was significantly higher ($P < 0.05$) compared to 0.1 mM JA treatment alone. After seven days of incubation, plants treated with the combination of 0.1 mM JA and four *T. urticae* for 2 days showed higher *PIOS* levels compared to control, 0.1 mM JA, and four *T. urticae* treatment alone ($P < 0.05$ for all comparisons). Compared to 0.1 mM JA or four *T. urticae* alone, the combination had a higher *PIOS* level than would be obtained from additive effects of four *T. urticae* and 0.1 mM JA, indicating a synergistic effect of the two treatments on *PIOS* transcript levels.

This experiment has been repeated two and three more times respectively and the results were consistent with those presented in Fig. 1. See Supplementary Fig. 1 and 2 for the results.

Phytohormone levels

We investigated the effects of single treatments i) water (control), ii) 0.1 mM JA, iii) four *T. urticae*, and iv) the combined treatment of 0.1 mM JA with simultaneous inoculation of four *T. urticae* on JA levels (Fig. 2). A significant treatment effect was found ($P = 0.01$; Fig 2A). Application of 0.1 mM JA resulted in higher JA levels at 48 h compared to control plants. Four *T. urticae*, however, did not increase JA levels in the plants compared to the control treatment. Plants treated with the combination of 0.1 mM JA and simultaneously four *T. urticae* also showed higher JA levels compared to control, but not different from 0.1 mM JA treatment alone.

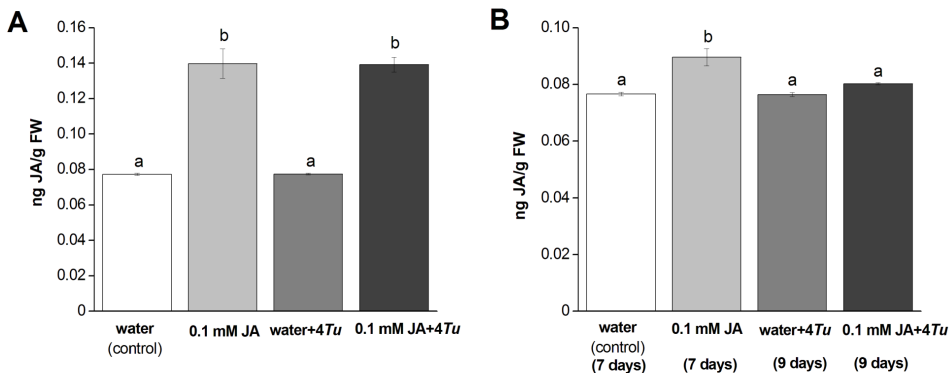


Fig. 2. JA levels in ng JA/g FW in *P. lunatus* plants treated with i) water (control), ii) 0.1 mM JA, iii) four *T. urticae* (water + 4*Tu*), or iv) 0.1 mM JA with four *T. urticae* mites (0.1 mM JA + 4*Tu*). (A) Plants were inoculated with four adult female *T. urticae* immediately after JA treatment and incubated for 48 h, and (B) plants were inoculated with four adult female *T. urticae* 7 days after JA treatment and incubated for an additional 48 h. Values are the mean (\pm SE) of four biological replicates, and were analyzed by Kruskal-Wallis test (A) or ANOVA (B) respectively ($\alpha = 0.05$).

Significant differences in JA levels were also found among treatments when mites had been inoculated seven days after JA or water application ($P < 0.01$; Fig 2B). After seven days of incubation with 0.1mM JA there is still an increase ($P < 0.001$) in JA level compared to control. The combination of 0.1 mM JA application and inoculation of *T. urticae* seven days later that had been feeding for two days resulted in JA levels after nine days that were similar to that of the control treatment. The introduction of four *T. urticae* alone did not affect JA levels.

No treatment effect was found for SA levels between control and other treatments for simultaneous ($P = 0.81$; Supplementary Fig. 3A) or sequential mite application ($P = 0.33$; Supplementary Fig. 3B).

Volatile emission

Emission rates of the monoterpene (*E*)- β -ocimene were compared among treatments and time of trapping of the simultaneous *T. urticae* application experiment. There was a treatment effect ($P < 0.05$), however, although emission rates of plants treated with 0.1 mM JA, mites, or both, were higher than control treatment, the post-hoc test did not yield statistical differences among treatments ($P > 0.05$; Fig. 3A). However, the time of trapping (morning, i.e. ca. 11.00 -

13.00 or afternoon, i.e. ca. 14.00 - 16.00) may also have an effect. Volatile trappings executed during mornings showed no overall effect of treatments ($P = 0.20$; Fig. 3B). In afternoon trappings, however, a treatment effect was found ($P = 0.02$; Fig. 3C), and plants treated with 0.1 mM JA and four *T. urticae* showed increased (*E*)- β -ocimene emission compared to other treatments ($P < 0.05$).

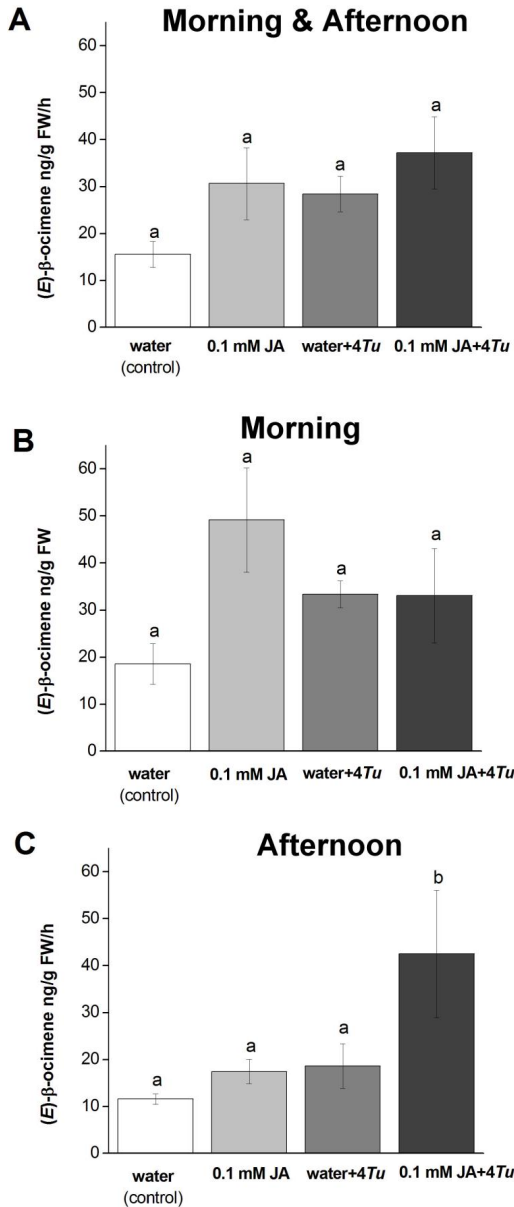


Fig. 3. Average (*E*)- β -ocimene emission rates in ng/g FW/h after four different treatments of *P. lunatus* plants. Treatments were i) control (water), ii) 0.1 mM JA, iii) four *T. urticae* (water + 4*Tu*), or iv) 0.1 mM JA with four *T. urticae* mites (0.1 mM JA + 4*Tu*) inoculated immediately after JA application and incubated for 48 h. (A) depicts combined morning and afternoon trappings, (B) morning trappings only (ca. 11.00 - 13.00), and (C) afternoon trappings only (ca. 14.00 - 16.00). Values are the mean (\pm SE) of six to seven biological replicates for (A), and three to four biological replicates for (B) and (C), except for water + 4*Tu* in (C) with two biological replicates. Different letters above bars indicate significant differences in emission rates between treatments (Fisher's LSD tests, $\alpha = 0.05$).

Emission of a total of the ten major volatile compounds was also compared among the treatments (Fig. 4). These ten compounds were (*E*)-2-hexenal, (*Z*)-3-hexen-1-ol, (*Z*)-3-

hexen-1-ol acetate, (*E*)- β -ocimene, linalool, methyl salicylate, indole, β -caryophyllene, (*E*)-DMNT, and (*E,E*)-TMTT. They constitute well-known herbivore-induced plant volatiles (HIPV) observed in *T. urticae*-infested Lima bean plants (Dicke *et al.*, 1990; Dicke *et al.*, 1999). PLS-DA including all four treatments resulted in a model with one significant component, whereby volatile blends emitted by control (water-treated) plants clearly differed from those emitted by plants exposed to the other three treatments. The volatile emission profiles of plants exposed to the combined 0.1 mM JA plus four *T. urticae* treatment overlapped to a large extent with those of plants exposed to 0.1 mM JA alone. Volatile blends emitted by plants exposed to four *T. urticae* exhibited similarities with those from control plants, but also with those from 0.1 mM JA-treated plants. Treatment of plants with JA, mites, or a JA-mite combination increased the emission of all ten volatiles (Fig. 4B). Compared to the control treatment, treatment of plants with JA (J and JT_u, Fig. 4B) resulted in higher emissions of indole, the green leaf volatiles (*Z*)-3-hexen-1-ol acetate and (*Z*)-3-hexen-1-ol, and to a lesser extent the terpenoids (*E*)-DMNT, (*E*)- β -ocimene, as well as β -caryophyllene. The emission rates of the latter three compounds were intermediate in plants exposed to mites alone.

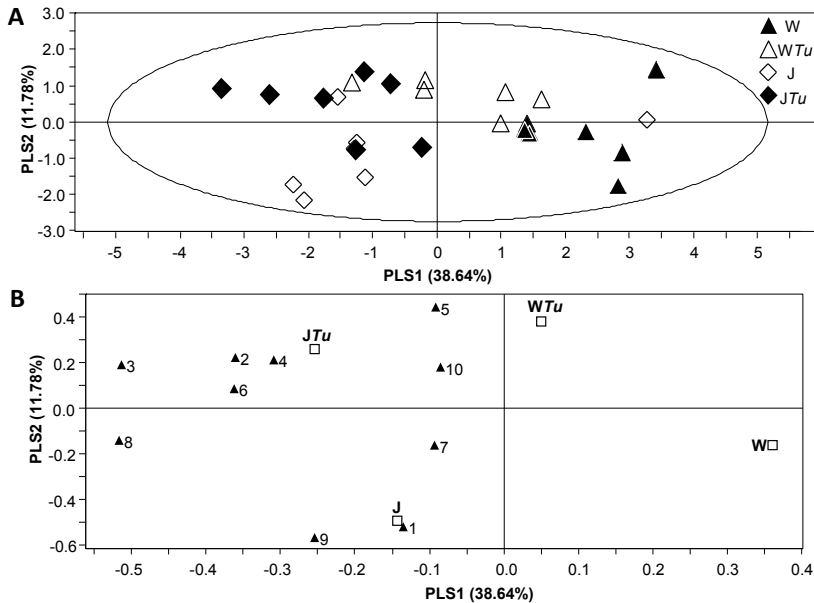


Fig. 4. Multivariate data analysis by PLS-DA and corresponding loading plot of targeted volatiles of *P. lunatus* plants exposed to i) water (control, W), ii) 0.1 mM JA (J), iii) water and four *T. urticae* spider mites (WT_u), or combined treatment iv) 0.1 mM JA with immediate application of four *T. urticae* (JT_u). (A) PLS-DA score plot showing the ordination of the samples according to the first two PLS components based on the quantitative values of volatiles between different treatments. Explained variance by first and second PLS components is given in brackets. Loading plot (B) shows the contribution of each volatile to the discrimination between treatments using the first two PLS components. Numbers represent: 1, (*E*)-2-hexenal; 2, (*Z*)-3-hexen-1-ol; 3, (*Z*)-3-hexen-1-ol acetate; 4, (*E*)- β -ocimene; 5, linalool; 6 (*E*)-4,8-dimethyl-1,3,7-nonatriene [(*E*)-DMNT]; 7, methyl salicylate (MeSA); 8, indole; 9, β -caryophyllene; 10, (*E,E*)-4,8,12-trimethyltrideca-1,3,7,11-tetraene [(*E,E*)-TMTT]. Squares represent the four treatments (labelled W, WT_u, J, and JT_u).

A pairwise comparison of volatile profiles from treatments including mites, i.e. water plus four *T. urticae* (WTu) and combined 0.1 mM JA treatment plus four *T. urticae* (JTU) resulted in a significant PLS-DA model with one significant component (Fig. 5). Pre-treatment with JA prior to *T. urticae* infestation resulted in a plant volatile profile that was separate from the profile of plants without the JA treatment.

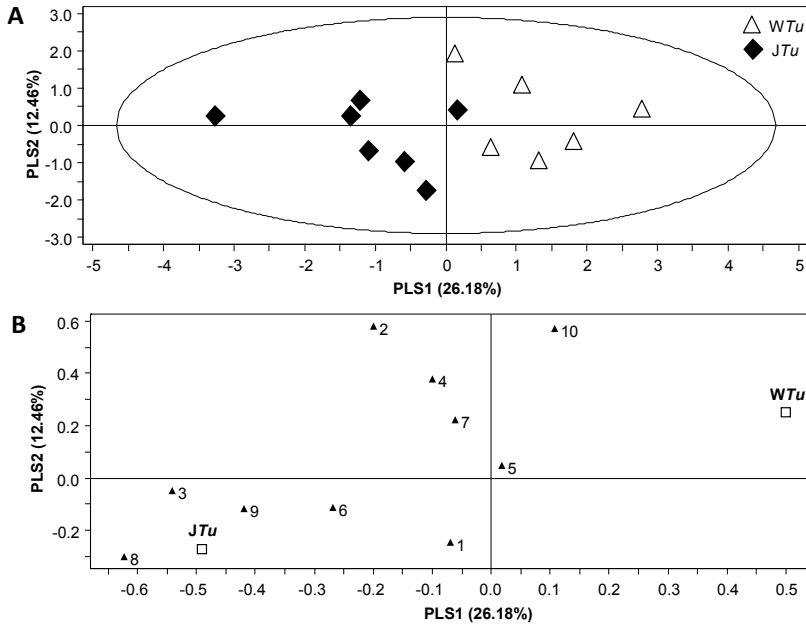


Fig. 5. Multivariate data analysis using PLS-DA and corresponding loading plot of volatile compounds emitted by *P. lunatus* plants subjected to either four *T. urticae* (WTu) or the combination of 0.1 mM JA and four *T. urticae* (JTU). The score plot (A) visualizes the separation pattern of the samples according to their classes using the first and second PLS component with the explained variance in brackets and the loading plot (B) depicts the contribution of volatiles to the class separation using the first two PLS components. The second PLS component was not significant and is only shown for representational purposes. Numbers represent: 1, (E)-2-hexenal; 2, (Z)-3-hexen-1-ol; 3, (Z)-3-hexen-1-ol acetate; 4, (E)- β -ocimene; 5, linalool; 6, (E)-4,8-dimethyl-1,3,7-nonatriene [(E)-DMNT]; 7, methyl salicylate (MeSA); 8, indole; 9, β -caryophyllene; 10, (E,E)-4,8,12-trimethyltrideca-1,3,7,11-tetraene [(E,E)-TMTT]. Squares represent the two treatments (labelled WTu and JTU).

Discussion

In their natural environment plants are frequently exposed to multiple herbivory, whereby herbivores may arrive simultaneously or separated in time. Both types of infestations may influence the plant phenotype and therefore affect tritrophic interactions with natural enemies involved in plant indirect defense. Here, we used the phytohormone JA followed by herbivory by a low number of herbivores to study the effects of this phytohormone on transcript levels of β -ocimene synthase, emission of the corresponding volatile compound, and other volatiles commonly emitted from plants in response to simultaneous and sequential herbivory. The volatile organic compound (*E*)- β -ocimene plays an important role in plant indirect defense in many plant species, including Lima bean, by attracting natural enemies of herbivorous arthropods (Dicke *et al.*, 1990; Arimura *et al.*, 2000; Arimura *et al.*, 2002; Zhang *et al.*, 2009a; Muroi *et al.*, 2011).

We found that Lima bean plants treated with a low dose of JA exhibited increased *PIOS* transcript levels in a synergistic manner when followed by minor herbivory, irrespective of the herbivory occurring simultaneously or sequentially. Accordingly, Gols *et al.* (2003) found that plants treated with a low dose of JA followed by simultaneous or sequential minor herbivory by *T. urticae* were highly attractive to the predatory mite *P. persimilis*: the predators preferred volatiles emitted from plants treated with 0.1 mM JA and infested with four *T. urticae* over volatiles from plants infested with only four *T. urticae*. Quantification of (*E*)- β -ocimene emission in the headspace of Lima bean plants shows that the emission rate of the volatile itself was also increased in combination treatments. The increase was only significant during the afternoon. The latter connects to findings of Arimura *et al.* (2008) that show that (*E*)- β -ocimene emission rates increase from the onset of light and peak during the afternoon after herbivory or leaf damage. Generally, (*E*)- β -ocimene seems to play an important role in the attraction of *P. persimilis* in plant interactions with multiple herbivores. For instance, De Boer *et al.* (2008) found that (*E*)- β -ocimene emission and predator attraction were increased in a synergistic manner in response to simultaneous infestation by prey and non-prey herbivores on a Lima bean plant. Moreover, Zhang *et al.* (2009b) showed that feeding by a non-prey herbivore, i.e. whiteflies, negatively affected (*E*)- β -ocimene emission and corresponding transcript levels of *PIOS*, which resulted in decreased attraction of *P. persimilis* to Lima bean plants simultaneously infested with spider-mites and whiteflies. The main underlying mechanism seems to be phytohormone induction and crosstalk among them. Whiteflies induce SA, which antagonizes the JA pathway, whereas caterpillars and spider mites mainly induce the JA pathway (Blechert *et al.*, 1995; Arimura *et al.*, 2002; Schmelz *et al.*, 2003). In our study we found a synergistic effect of a low dose 0.1 mM JA and a low density infestation by four *T. urticae* on *PIOS* transcript levels after 48 h of spider-mite infestation. In the case of a 7-day delay between JA treatment and spider-mite inoculation JA did not induce *PIOS* transcription but in combination with spider mite feeding resulted in an enhanced transcription compared to spider-mite induction alone. Thus, in this case JA had primed the transcription of this gene. Yet JA levels were similar for JA-treated plants and plants induced with both JA and *T. urticae*. Interestingly, even when JA titers and *PIOS* transcripts levels returned to control levels, subsequent mite infestation still increased *PIOS* transcript levels to higher values than

recorded after mite infestation alone. Introduction of a time lag between first induction of plant defense by JA and a second induction by herbivory did not impair plant ability for enhanced defense induction. In fact, this corresponds with behavioral results reported by Gols *et al.* (2003) for the predatory mite *P. persimilis*, which was more strongly attracted to sequentially induced plants than to plants only induced by spider mites. It has been previously suggested that plants are able to form some sort of memory, sometimes called a “primed state”, which enables them to accelerate and/or enhance defense responses to a second challenge (Frost *et al.*, 2008; Conrath, 2009). Maintenance of plant defense is thought to entail costs and is ineffective in the absence of herbivores. Consequently, plants have developed defense mechanisms that are inducible by herbivory (Heil and Baldwin, 2002). In the case of priming, costly defense metabolites are not produced immediately upon a minor challenge, thereby considerably reducing the cost of this mechanism (Van Hulten *et al.*, 2006; Walters *et al.*, 2008; Perazzolli *et al.*, 2011). In our experiments, previous induction of *PIOS* by JA seemed to sensitize the gene in such a way that a second challenge using a small number of herbivores at a later time point resulted in increased transcript levels. The ability of phytohormones to generate a primed state in terms of enhanced defense gene transcription has previously been reported for e.g. SA and the SA-analogue benzothiadiazole (BTH) in *Petroselinum crispum* L. and *Arabidopsis thaliana* (Thulke and Conrath, 1998; Kohler *et al.*, 2002).

Natural enemies of herbivores respond to mixtures of HIPV rather than to a single volatile. Blends can carry information on e.g. herbivore identity or herbivore developmental stage (Takabayashi *et al.*, 1995; De Moraes *et al.*, 1998; Stout *et al.*, 1998; De Vos *et al.*, 2005; Mumm and Dicke, 2010). JA application is known to induce a volatile blend that is similar to the blend induced by *T. urticae* mites (Dicke *et al.*, 1999; Gols *et al.*, 1999). However, defense induction by JA seems to be more generic and natural enemies often prefer HIPVs induced by actual hosts or prey over JA-induced plants (Van Poecke and Dicke, 2002; De Boer and Dicke, 2004; Ozawa *et al.*, 2004; Bruinsma *et al.*, 2008; Bruinsma *et al.*, 2009b). Our targeted chemical analysis comparing the volatile profiles of 10 well-known major HIPVs emitted by Lima bean plants among treatments showed indeed a large overlap for JA- and mite-treated plants and a clear separation from the blend emitted by control plants. However, Gols *et al.* (2003) found that volatiles emitted by Lima bean plants in response to a low dose of 0.1 mM JA do not attract the predator *P. persimilis*, whereas a low infestation density of four *T. urticae*, and particularly the combination of treatments, does. Qualitative and quantitative differences in volatile blends must thus affect the behavior of the predatory mite. Volatile emission profiles of plants with herbivores with and without simultaneous JA treatment do not only show a great overlap, but also demonstrated that other volatiles, besides (*E*)- β -ocimene, are likely to determine attractiveness of the volatile blend attractive to *P. persimilis*. While (*E*)- β -ocimene is known to be an important host location cues in Lima bean, De Boer *et al.* (2004) found that (*E*)- β -ocimene is also emitted in response to caterpillar feeding. Predators must therefore gain additional information from other HIPVs, such as MeSA and (*E,E*)-TMTT, in order to distinguish prey-infested plants from non-prey infested plants.

Conclusion

Application of a low dose of the phytohormone JA results in augmented transcript levels of a terpene biosynthetic gene and emission of a volatile metabolite crucial in plant indirect defense, when followed by a minor infestation of herbivores. This synergistic effect is observed irrespective of whether phytohormone and infestation occur simultaneously or sequentially, and might lead to a memory effect of plant indirect defense. Phytohormone application has thus the potential to induce enhanced biological pest control against spider mites. Moreover, this study provides information that indirect defense is stable in case of simultaneous and sequential attack by herbivores that induce similar signal transduction pathways in plants and may even be enhanced in the presence of multiple herbivores. However, the effect on other tritrophic interactions, other plants species, and the persistence of this effect require further investigation.

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Chapter 4
**Transcriptional responses of Lima
bean
defense genes to feeding or oral
secretions of *Mamestra brassicae*
L. and *Spodoptera exigua* Hübner
caterpillars**

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Abstract

Recognition of herbivorous attackers comprises an integral part of plant defense and is essential for an adequate defense response to a specific attacker. Caterpillar feeding as well as application of caterpillar oral secretion on mechanical wounds, are frequently used to induce plant defense against biting-chewing insects. Here, we used feeding damage or the combination of mechanical damage and oral secretions of the generalist caterpillar species *Mamestra brassicae* and *Spodoptera exigua* to study the damage- and species-specific effects on transcript levels of three defense-related genes in Lima bean (*Phaseolus lunatus*), i.e. *P. lunatus* lipoxygenase (*PILOX*), *P. lunatus* β -ocimene synthase (*PIOS*), *P. lunatus* acidic pathogenesis-related protein 4 (*PIPR-4*). Since induction of defense can affect subsequent herbivores, we also investigated how the induction of defense genes by feeding damage or mechanical damage plus oral secretion affected the defense response to subsequent herbivory by the spider mites *Tetranychus urticae*. While patterns of gene transcription were mostly identical in response to the two caterpillar species, feeding damage or mechanical damage plus caterpillar oral secretion caused differential induction of the transcription of defense genes. Nevertheless, compared to plants with single herbivory, plants with dual herbivory only showed differential gene induction for *PIPR-4*. Lima bean plants respond differently to caterpillar feeding than to mechanical damage plus caterpillar oral secretion, resulting in different effects on plant direct and indirect defense against subsequent herbivores.

Introduction

Plants are frequently attacked by herbivorous arthropods, which can cause considerable detrimental effects on plant fitness. Consequently, plants have developed sophisticated defense mechanisms ranging from morphological structures to the production of toxic compounds (Schoonhoven *et al.*, 2005; Hanley *et al.*, 2007). Plant defenses can be constitutive or induced, the latter only being activated in response to attack. Plant defense mechanisms are considered quite costly in the absence of an attacker due to allocation costs associated with mounting defenses (Strauss *et al.*, 2002). This is the result of a trade-off between plant defense and plant growth and reproduction (Baldwin *et al.*, 1990; Herms and Mattson, 1992). Induced defense can be very specific and provide adaptive advantage in comparison to constitutive defense (Karban *et al.*, 1997). Plants possess a sophisticated and well-orchestrated signaling network that results in responses that are specific for different attacker species (e.g. De Vos *et al.*, 2005). This system allows plants to mount an effective defense to herbivorous arthropods as well as to other attackers such as pathogens. Furthermore, it has been shown that plants can convey information on the attack status to beneficial insects, such as natural enemies of herbivores, and distant tissues or other plants (Rodriguez-Saona *et al.*, 2009; Mumm and Dicke, 2010; Ramadan *et al.*, 2011). The information conveyed can relate to herbivore species and developmental stage, which suggests that plants can discriminate between different attackers (Takabayashi *et al.*, 1995; De Moraes *et al.*, 1998).

In fact plants are known to activate distinct defense pathways in response to herbivores belonging to different feeding guilds. The main defense pathways are the octadecanoid pathway, shikimate pathway, and ethylene pathway with jasmonic acid (JA), salicylic acid (SA), and ethylene (ET) as their respective signaling molecules. There is antagonistic crosstalk between the octadecanoid and the shikimate pathways, which is thought to play an important role in fine tuning plant defense (Koornneef and Pieterse, 2008; Wei *et al.*, 2014). It has been hypothesized that plants distinguish herbivores based on their feeding modes. Piercing-sucking herbivores, such as whiteflies, mainly induce SA and leaf-chewing herbivores and cell content feeders, such as caterpillars and spider mites, induce especially JA (Kempema *et al.*, 2007; Stam *et al.*, 2014). Plants can recognize attackers through elicitors released by herbivores during activities such as feeding and oviposition, (reviewed in (Hilker and Meiners, 2010; Bonaventure *et al.*, 2011). Elicitors isolated from caterpillar oral secretions identified thus far are structurally diverse, comprising fatty acid amino acid conjugates (FACs) and enzymes, and have been used to effectively mimic feeding of caterpillars (e.g. (Turlings *et al.*, 1990; Mattiacci *et al.*, 1995; McCloud and Baldwin, 1997; Roda *et al.*, 2004; Schmelz *et al.*, 2007).

Here we investigated the effect of feeding or a combination of standardized mechanical damage and application of oral secretions to the wounded tissue of two generalist caterpillars on the induction of Lima bean defense genes. Moreover, we investigated the effect on the same genes when induction by the caterpillar treatments was followed by infestation by a second herbivore, the generalist spider mite *Tetranychus urticae*. Herbivory by caterpillars and spider mites is known to induce JA-related defense (Li *et al.*, 2002; Stam *et al.*, 2014).

Phaseolus lunatus lipoxygenase (*PILOX*) and *Phaseolus lunatus* β -ocimene synthase (*PIOS*) are two JA-inducible genes (Arimura *et al.*, 2000; Arimura *et al.*, 2008). The gene *PILOX* is located early in the octadecanoid pathway, while *PIOS* is located downstream and codes for a rate-limiting step in the biosynthesis of the inducible plant volatile (*E*)- β -ocimene, which is known to attract the predatory mite *Phytoseiulus persimilis*, a natural enemy of *T. urticae* (Dicke *et al.*, 1990; Ament *et al.*, 2004). The gene *PIPR-4* codes for an acidic chitinase, which is methyl salicylate-responsive (Margis-Pinheiro *et al.*, 1991; Arimura *et al.*, 2000). We hypothesized that caterpillars would equally upregulate JA defenses, regardless of induction method and species, and that caterpillar pre-treatment would have a positive effect on plant defense against the second herbivore, *T. urticae*.

Materials and Methods

Plants and insects

Lima bean plants (*Phaseolus lunatus* L., cv Wonderbush) were sown and grown in a greenhouse compartment at 23 ± 2 °C with 60 ± 10 % R.H., and a photoperiod of 16L : 8D. Plants were used for experiments 12 - 15 days after sowing, when their primary leaves had fully expanded. For the duration of experiments, plants were kept in a climate chamber and incubated at 25 ± 1 °C, 60 ± 10 % R.H. and 16L : 8D. In the climate chamber, plants of different treatments were kept separate in metal-frame cages with polyethylene sheet walls (90 x 90 x 60 cm). Each cage was connected to the house vacuum to prevent volatile transfer between plants with different treatments.

Two-spotted spider mites, *Tetranychus urticae* Koch (Acari: Tetranychidae), were reared on Lima bean plants in a different greenhouse compartment under the same conditions as the Lima bean plants. Randomly selected adult female mites were used for experiments.

Cabbage moth, *Mamestra brassicae* L. (Lepidoptera: Noctuidae) and beet armyworm, *Spodoptera exigua* Hübner (Lepidoptera: Noctuidae), caterpillars were reared on cabbage plants (*Brassica oleracea* var. gemmifera cv. Cyrus, Syngenta seeds BV, Enkhuizen, The Netherlands) at 22 ± 1 °C and 50 - 70 % R.H., under the same photoregime as the Lima bean plants. Prior to experiments *S. exigua*, but not *M. brassicae*, caterpillars were transferred to feed on Lima bean plants for 24 h. Oral secretion of each caterpillar species was collected from 20 randomly selected caterpillars in the 5th instar.

For feeding treatments, eggs of *S. exigua* and *M. brassicae* were obtained from the general cultures in our laboratory (Smits *et al.*, 1986; Menzel *et al.*, 2014). Paper sheets containing the egg batches were then placed on Lima bean plants and kept in a climate chamber at 22 ± 1 and 50 - 70 % R.H. Once caterpillars hatched, Lima bean plants were added as food source if necessary and larvae in the 3rd instar were used for feeding experiments.

Treatments

Effects of feeding and mechanical damage plus oral secretion of the two caterpillar species

Plant treatments consisted of i) control ii) mechanical damage iii) caterpillar feeding by *M. brassicae* or *S. exigua* iv) mechanical damage plus caterpillar oral secretion of *M. brassicae* or *S. exigua* respectively. Plants of all treatments received one clip cage (diam. 2.5 cm) supported by sticks per primary leaf. Control plants i) did not receive further treatment until sample collection. For treatment ii), plants were artificially damaged using a pattern wheel to draw 6 lines of ca. 7 cm length on the primary leaves and then incubated until sample collection. For treatment iii) two L3 caterpillars of either species were inoculated, each confined in a separate clip cage on a primary leaf. Treatment iv) consisted of artificially damaging the plants in the same manner as for treatment ii) and then applying caterpillar oral secretion onto the wounds. Oral secretion consisted of a pooled stock collected from 20 caterpillar of either caterpillar species. The stock was diluted 1:1 with tap water and kept on ice before application. Using a pipette, 10 µl of the diluted caterpillar oral secretion stock was applied onto the wounds inflicted by the pattern wheel, and distributed using a fine paint brush. Plants of all treatments were incubated for 48 h. Clip cages and caterpillars were removed shortly before sample collection and plants were carefully cleaned from feces with a fine paint brush.

*Effects of *M. brassicae* feeding and oral secretion on subsequent herbivory*

Plant treatments consisted of i) control, ii) caterpillar feeding followed by a period without infestation, iii) *T. urticae* infestation, iv) caterpillar feeding followed by infestation with *T. urticae*, v) incubation with caterpillar oral secretion followed by infestation with *T. urticae*. All plants received one clip cage (diam. 2.5 cm) per primary leaf. Control plants i) did not receive further treatment. For treatment ii) one *M. brassicae* L3 caterpillar per clip cage was inoculated on the primary leaves. After 48 h caterpillars and their feces were carefully removed using a fine paint brush and plants were subsequently incubated for another 48 h without herbivores. For treatment iii) plants were also exposed to *M. brassicae* caterpillars that fed for 48 h, but immediately after removal of caterpillars, 20 female *T. urticae* were evenly distributed over the two primary leaves. After 48 h of infestation *T. urticae*, their webbing, and feces were removed with a fine paint brush. Treatment iv) was done by inflicting artificial damage and application of 10 µl of diluted *M. brassicae* oral secretion as described above. After 48 h of incubation, 20 female *T. urticae* were evenly distributed over the primary leaves. Mites, their webbing, and feces were removed with a fine paint brush after 48 h of infestation. Treatment v) consisted of a 48 h infestation by 20 female *T. urticae* that were evenly distributed over the two primary leaves.

RNA extraction and cDNA synthesis

Plant material was collected and processed as previously described in Menzel *et al.* (2014). In short, four biological replicates were collected for each treatment per experiment. Each biological replicate consisted of plant leaf material pooled from primary leaves of three plants. Experiments were repeated up to seven times, whereby not all treatments were included in each experiment.

Quantitative RT-PCR

To quantify transcript levels of *P. lunatus* β -ocimene synthase (*PIOS*; GenBank accession EU194553), *P. lunatus* acidic pathogenesis-related protein 4 (*PIPR-4*), *P. lunatus* lipoxygenase (*PILOX*; GenBank accession X63521), and the two reference genes *P. lunatus* Actin1 (*PIACT1*; GenBank accession DQ159907) and *P. lunatus* Nuclear matrix protein 1 (*PINMP1*; GenBank accession AF289260.1), real-time quantitative RT-PCR was performed in a Rotor-Gene 6000 machine (Corbett Research) with a 72-well rotor. For a detailed description refer to Menzel *et al.* (2014). *PIOS* primers were F-*PIOS* 5'-TGCATGGGTCTCAGTCTCTG-3' and R-*PIOS* 5'-TGCTGCTTCCCCTCTCTCTA-3', *PIPR-4* were F-*PIPR-4* 5'-ACGCTTTCCTCAGTGCTCTC-3' and R-*PIPR-4* 5'-TCCTCGTCGTCGCAGTAATCCTT-3', *PILOX* primers were F-*PILOX* 5'-GGAATGGGACAGGGTTTATG-3' and R-*PILOX* 5'-CAAAGTCACTGGGCTTCTCA-3', *PIACT1* primers were F-*PIACT1* 5'-CCAAGGCTAACCGTGAAAAG-3' and R-*PIACT1* 5'-AGCCAGATCAAGACGAAGGA-3', and *PINMP1* primers F-*PINMP1* 5'-CCGGAATGGAGTGTTGACGAGCA-3' and R-*PINMP1* 5'-CCAGCTCAGAAACATCTGGCAATGG-3'. Gene transcripts were quantified using the 2(-Delta Delta Ct) method (Livak and Schmittgen, 2001), using a normalization factor (Vandesompele *et al.*, 2002). The latter was calculated by geometrically averaging the threshold cycle (Ct) values from the two reference genes *PIACT1* and *PINMP1*. Subtraction of the normalization factor from Ct values normalizes for differences in cDNA synthesis.

Statistical analysis

Gene transcription data were log-transformed and analyzed by one-way ANOVA followed by Tukey's Honestly Significant Difference (HSD) for pairwise comparisons between treatments in the statistical software SPSS version 19 (SPSS Inc., Chicago, IL, USA). If assumptions on normality and equal variance were violated, data were analyzed by Kruskal-Wallis tests followed by Mann-Whitney U tests with a Bonferroni correction as post-hoc tests.

Results

Effects of feeding or mechanical damage plus oral secretion of the two caterpillar species on gene transcription

Relative quantification of *PIOS* transcripts showed that treatments significantly affected *PIOS* transcript levels (Kruskal Wallis test for both caterpillar species $P < 0.01$; Fig. 1). Plants treated with mechanical damage or mechanical damage plus caterpillar oral secretion did not show significant differences in *PIOS* transcript levels compared to control plants or when compared to each other. In contrast, feeding by two *M. brassicae* or *S. exigua* caterpillars resulted in significantly higher *PIOS* transcript levels compared to control plants and other treatments (Fig. 1).

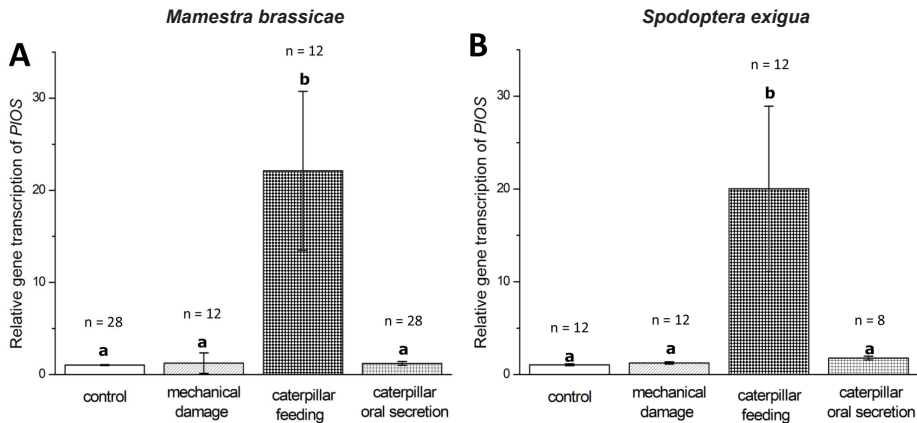


Fig. 1. Relative gene transcript levels of *P. lunatus* (*E*)- β -ocimene synthase (*PIOS*). Plant treatments were i) no treatment (control), ii) mechanical damage at 48 hours post treatment (hpt) (mechanical damage), iii) caterpillar feeding for 48 h (caterpillar feeding), and iv) mechanical damage plus application of caterpillar oral secretion at 48 hpt (caterpillar oral secretion). Values are the mean (\pm SE) of 8-28 biological replicates (n). Different letters above bars within a panel indicate significant differences in transcript levels between treatments (Kruskal Wallis test followed by Mann-Whitney U tests with Bonferroni correction, $P < 0.05$).

Relative gene transcript levels of *PILOX* were also significantly affected by the different treatments (ANOVA, $P < 0.001$ for both caterpillar species; Fig. 2). All three treatments, namely mechanical damage, caterpillar feeding, and mechanical damage with caterpillar oral secretion led to increased *PILOX* transcript levels compared to the levels in control plants (post hoc Tukey's HSD for all pairwise comparisons $P < 0.001$). However, the treatments showed no significant differences in transcript levels amongst each other.

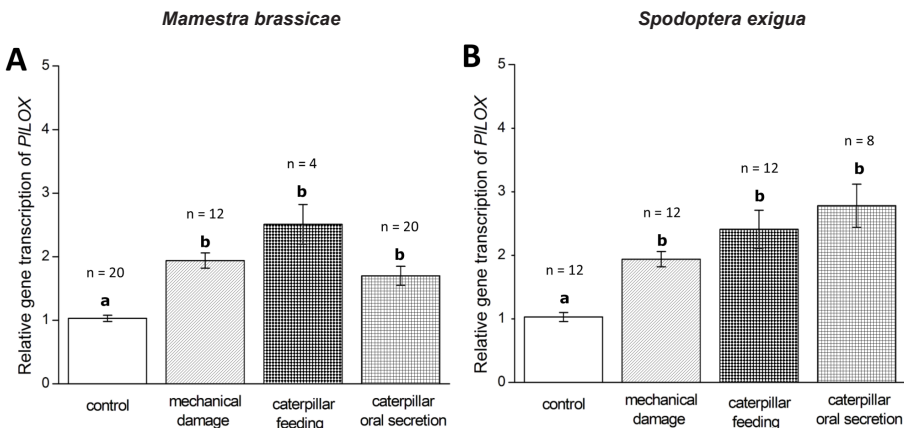


Fig. 2. Relative gene transcript levels of *P. lunatus* lipoxygenase (*PILOX*). Plant treatments were i) no treatment (control), ii) mechanical damage at 48 hours post treatment (hpt) (mechanical damage), iii) caterpillar feeding for 48 h (caterpillar feeding), and iv) mechanical damage with caterpillar oral secretion at 48 hpt (caterpillar oral secretion). Values are the mean (\pm SE) of 4-20 biological replicates (n). Different letters above bars within a panel indicate significant differences in transcript levels between treatments (ANOVA followed by post hoc Tukey's HSD test, $P < 0.05$).

Transcript levels of *PIPR-4* were significantly affected by *M. brassicae* and *S. exigua* treatments (Fig. 3, Kruskal Wallis test, $P < 0.01$). Whereas mechanical damage and caterpillar feeding did not significantly alter transcript levels compared to control levels, the application of *M. brassicae* and *S. exigua* caterpillar oral secretion resulted in an increase of *PIPR-4* levels (Kruskal Wallis test followed by Mann-Whitney U tests with Bonferroni correction, $P \leq 0.001$). The transcript level induced by mechanical damage plus *M. brassicae* oral secretion treatment was not significantly different from levels found in plants fed upon by *M. brassicae* or mechanical damage alone. In contrast, the *PIPR-4* transcript level induced by *S. exigua* oral secretion treatment was significantly different from levels found in plants that had only been mechanically damaged, but also not different from plants fed upon by *S. exigua*.

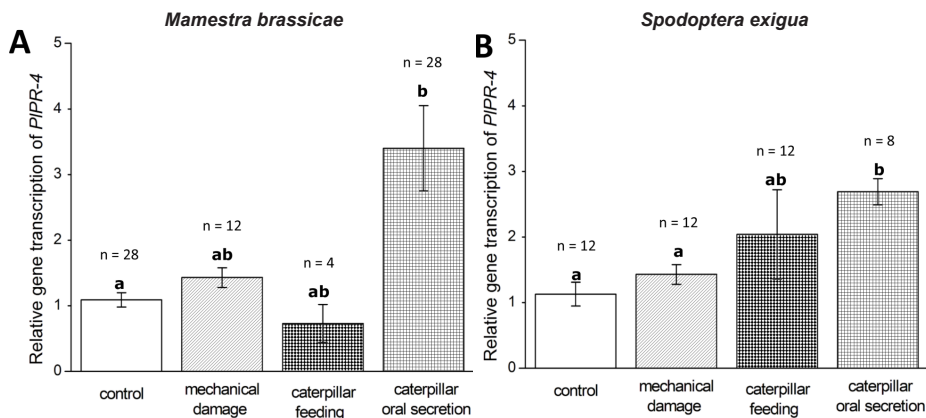


Fig. 3. Relative gene transcript levels of *P. lunatus pathogenesis related protein 4* (*PIPR-4*). Plant treatments were i) no treatment (control), ii) mechanical damage at 48 hours post treatment (hpt) (mechanical damage), iii) caterpillar feeding for 48 h (caterpillar feeding), and iv) mechanical damage with caterpillar oral secretion at 48 hpt (caterpillar oral secretion). Values are the mean (\pm SE) of 4-28 biological replicates (n). Values within a panel having no letters in common above the bar were significantly different (Kruskal Wallis test followed by Mann-Whitney U tests with Bonferroni correction, $P < 0.05$).

Effects of *Mamestra brassicae* feeding or oral secretion application on gene induction by subsequent spider mite herbivory

Relative transcript levels of *PIOS* and *PIPR-4* were significantly affected by treatments (Fig. 4, ANOVA, $P < 0.001$ for both genes). Plants infested for 48 h by *T. urticae* showed significantly increased *PIOS* transcript levels compared to control, irrespective of whether they had received a prior treatment with *M. brassicae* feeding or oral secretion (post hoc Tukey's HSD, $P < 0.001$). However, there was no significant difference among *T. urticae* treatments, thus prior caterpillar treatments did not affect *PIOS* transcript levels compared to a *T. urticae* infestation alone (post hoc Tukey's HSD, $P > 0.05$). Transcript levels of plants which had been fed upon by *M. brassicae* followed by 48 h without herbivory, showed transcript levels similar to those of control plants for *PIOS* and *PIPR-4*. Yet, prior treatment of plants with *M. brassicae* oral secretion resulted in higher *PIPR-4* transcript levels after spider mite treatment compared to plants treated with *T. urticae* infestation alone (post hoc Tukey's HSD, $P < 0.01$). Relative transcript levels of *PILOX* were not significantly affected by treatments (Fig. 4 B).

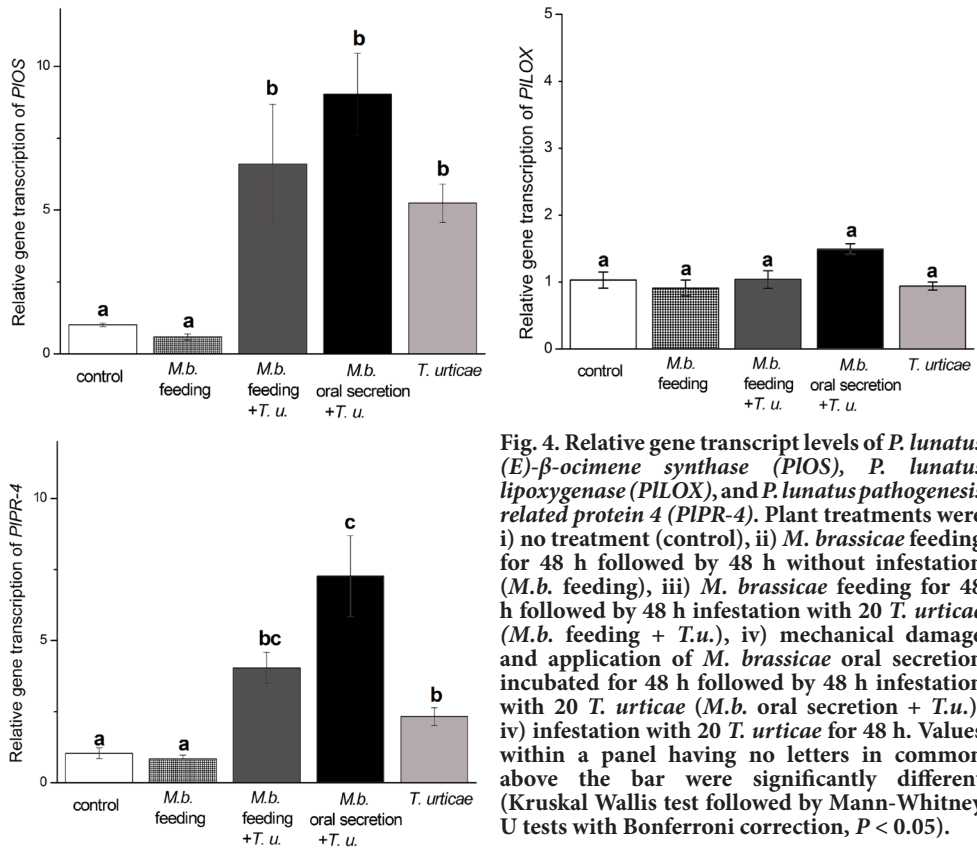


Fig. 4. Relative gene transcript levels of *P. lunatus* (E)- β -ocimene synthase (PIOS), *P. lunatus* lipooxygenase (PILOX), and *P. lunatus* pathogenesis related protein 4 (PIPR-4). Plant treatments were i) no treatment (control), ii) *M. brassicae* feeding for 48 h followed by 48 h without infestation (*M.b.* feeding), iii) *M. brassicae* feeding for 48 h followed by 48 h infestation with 20 *T. urticae* (*M.b.* feeding + *T.u.*), iv) mechanical damage and application of *M. brassicae* oral secretion incubated for 48 h followed by 48 h infestation with 20 *T. urticae* (*M.b.* oral secretion + *T.u.*), iv) infestation with 20 *T. urticae* for 48 h. Values within a panel having no letters in common above the bar were significantly different (Kruskal Wallis test followed by Mann-Whitney U tests with Bonferroni correction, $P < 0.05$).

Discussion

Plant defenses are fine-tuned to such an extent that plants respond differently depending on herbivore species, life stage, and condition. It has been previously shown that oral secretions of a broad range of lepidopterans contain FACs, which are known as potent inducers of plant direct and indirect defenses (Roda *et al.*, 2004; Yoshinaga *et al.*, 2010). Here we investigated the effects of caterpillar feeding and the application of oral secretion of two generalist caterpillar species on gene transcription of relevant defense genes in Lima bean plants that are involved in defense signaling and synthesis of defense metabolites. Our results showed that caterpillar species did not cause differential effects on gene transcription of *PIOS*, *PILOX* or *PIPR-4*, suggesting that plants did not distinguish between species. Interestingly, caterpillar feeding and application of caterpillar oral secretion exerted significantly different effects on gene transcript levels. Whereas oral secretions did not increase *PIOS* transcripts above control levels, caterpillar feeding caused a strong increase in *PIOS* transcript levels. The latter is in accordance with findings by De Boer *et al.* (2008) who found that feeding by *S. exigua* causes an increase in the corresponding volatile (*E*)- β -ocimene in Lima bean plants. The monoterpene (*E*)- β -ocimene plays an important role in plant indirect defense in many plant species (Dicke *et al.*, 1990; Arimura *et al.*, 2000; Zhang *et al.*, 2009; Muroi *et al.*, 2011). It is likely that the observed difference in *PIOS* levels for these treatments were caused by the temporal differences in defense stimulation by mechanical damage plus oral secretion application and continuous feeding. Continuous feeding by caterpillars results in a repeated stimulation of plant defense via removal of plant tissue and thereby repeated application of oral secretion (Vadassery *et al.*, 2012). In contrast, in our experiments mechanical damage with application of caterpillar oral secretion consisted of a single stimulation of defense which may subside quickly. Mithöfer *et al.* (2005) showed that rhythmic leaf removal was necessary to induce a volatile profile that closely resembles the volatile profile induced by *S. littoralis* feeding. Their study also showed that this was not the case for mechanical damage induced by one-time damaging with a pattern wheel; however, one-time mechanical damage was not combined with caterpillar oral secretion application in their study. Moreover, we can exclude the possibility that oral secretion was inactive or did not contain elicitors because *PIPR-4* was significantly higher induced in plants that had received caterpillar oral secretion from *S. exigua* compared to mechanical damage. Furthermore, caterpillar feeding and mechanical damage, either or not combined with application of oral secretions to the damaged tissue all induced *PILOX*, a gene which is located early in the octadecanoid pathway and necessary for wound-induced JA accumulation (Bell *et al.*, 1995). This suggests that JA and the octadecanoid pathway were indeed induced by all these treatments, leading to a generic wound response. However, *PIOS*, downstream the JA-signaling cascade, was activated only by caterpillar feeding at the time point investigated. It is less likely that the observed differences in our study were caused by the different diets of caterpillars used for feeding damage and oral secretion collection as it has been shown previously that the induction of defense and relative amounts of conjugates is diet and instar independent (Turlings *et al.*, 1993; Pohnert *et al.*, 1999).

Effect of *M. brassicae* feeding or mechanical damage plus oral secretion application on gene induction by subsequent spider mite herbivory

In nature, plants are often attacked by more than one herbivore, whereby the defense induction by the first herbivore can have a significant effect on the resistance against a second herbivore (e.g. (Kessler and Baldwin, 2004; De Boer *et al.*, 2008; Dicke *et al.*, 2009; Zhang *et al.*, 2009). In our study, defense induction by caterpillar feeding or oral secretion which was followed by herbivory by the mite *T. urticae* did not show differences in defense response compared to single herbivory by the mite for *PIOS* or *PILOX*, but only for *PIPR-4*. De Boer *et al.* (2008) found a synergistic effect of simultaneous feeding of *S. exigua* and *T. urticae* on Lima bean indirect defense against *T. urticae*. Since lepidopterans, and certain cell-content feeders, such as *T. urticae*, mainly induce the octadecanoid pathway, and also volatile induction by both herbivores is regulated by this pathway, such an effect can be expected (Ozawa *et al.*, 2000). In our experiments, however, we used not only lower numbers of herbivores, but plants also received herbivore damage sequentially rather than simultaneously. Nevertheless, Choh and Takabayashi (2010) found that *T. urticae* mites avoid leaves previously fed upon by the caterpillar *Spodoptera litura* F. and mite oviposition was reduced on plants with *S. litura* compared to control plants. This suggests that herbivory by caterpillars can also induce resistance against *T. urticae* via direct defense mechanisms. In fact, we found an upregulation of *PIPR-4* transcript levels when plants were treated with oral secretion before *T. urticae* infestation. The gene *PIPR-4* codes for a methyl salicylate-responsive acidic chitinase (Margis-Pinheiro *et al.*, 1991; Arimura *et al.*, 2000). Chitinases are digestive enzymes that degrade glycosidic bonds in chitin, which makes up the cell walls of plant pathogens and is a major component of the peritrophic membrane in the gut of herbivorous arthropods (Schlumbaum *et al.*, 1986; Kramer and Muthukrishnan, 1997). Nevertheless, *PIPR-4* transcript levels were not significantly upregulated at the observed time point in plants that experienced caterpillar feeding. The latter confirms that there are differences in defense gene induction between caterpillar oral secretions and caterpillar feeding, which influence the defense response to a second herbivore.

The fact that transcript levels of *PILOX* were not affected by the treatments, might be due to its upstream location in the octadecanoid pathway. It has been suggested that early defense signaling includes phytohormone-mediated defense pathway activation, while later responses include the activation of more specific defense gene induction and defense metabolites (Stam *et al.*, 2014).

Conclusion

Plants differentially respond to caterpillar feeding and the application of caterpillar oral secretion. The two caterpillar species had a similar effect on defense response in terms of gene induction. Nevertheless, when defense induction was followed by infestation by a second herbivore gene expression appeared not to be affected by the differences in defense induction by caterpillar feeding and application of caterpillar oral secretion.

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Chapter 5
**Effect of sequential induction by
Mamestra brassicae L. and *Tetranychus*
urticae Koch on Lima bean plant
indirect defense**

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Abstract

Attack by multiple herbivores often leads to modification of induced plant defenses compared to single herbivory, yet little is known about the effects on induced indirect plant defense. Here, we investigated the effect of sequential induction of plant defense by *Mamestra brassicae* caterpillar oral secretion and an infestation by *Tetranychus urticae* spider mites on the expression of indirect plant defense in Lima bean plants. The effect on indirect defense was assessed using behavior assays with the specialist predatory mite *Phytoseiulus persimilis* in an olfactometer, headspace analysis of 11 major herbivore-induced plant volatiles (HIPVs) including (*E*)- β -ocimene and transcript levels of the corresponding gene *Phaseolus lunatus* β -ocimene synthase (*PIOS*). Predatory mites were found to distinguish between plants induced by spider mites and caterpillar oral secretion but not between plants with single spider mite infestation and plants induced by caterpillar oral secretion prior to spider mite infestation. Indeed, the volatile blends emitted by plants induced by spider mites only and the sequential induction treatment of caterpillar oral secretion followed by spider mite infestation, were similar. Our results suggest that plant indirect defense is not affected by previous treatment with oral secretion of *M. brassicae* caterpillars.

Introduction

Plants that are under attack by herbivores produce and release complex mixtures of volatiles, known as herbivore-induced plant volatiles (HIPVs). Natural enemies of herbivorous arthropods can use these HIPVs to locate their herbivorous prey or host (reviewed by Mumm and Dicke (2010)). The phenomenon of recruitment of natural enemies via HIPV release is known as indirect plant defense. Phytohormones, such as jasmonic acid (JA), salicylic acid (SA) and ethylene (ET) are involved in generating and modulating induced plant defense responses to herbivory. The phytohormone JA and its volatile derivative methyl jasmonate (MeJA) are the main signaling molecules for induction of plant defense against herbivores and play an important role in indirect plant defense (e.g. Dicke *et al.* 1999; Kessler and Baldwin 2002). However, other phytohormones, such as salicylic acid (SA) and ethylene (ET) are known to play modulating roles via pathway cross-talk (Ozawa *et al.*, 2000; Van Poecke and Dicke, 2002; Ament *et al.*, 2004; Koornneef and Pieterse, 2008). Depending on the signaling pathways induced by an herbivore, the composition of HIPV blends can differ significantly (Zhang *et al.*, 2013), allowing natural enemies to distinguish between plants infested by prey and non-prey herbivores (De Boer *et al.*, 2004; Erb *et al.*, 2010).

In nature, prey and non-prey herbivores can simultaneously feed on the same plant and thereby differentially modulate defense pathways and alter plant defense responses. Multiple herbivory is subject to an increasing number of studies (Stam *et al.*, 2014). However, most of these studies focus on the effect on direct plant defenses and plant-mediated interactions amongst the herbivores (Kaplan and Denno, 2007; Brunissen *et al.*, 2009; Rodriguez-Saona *et al.*, 2010; Erb *et al.*, 2011; Mathur *et al.*, 2013). The few studies on indirect plant defense mechanisms (Dicke *et al.*, 2009) show that volatile blends of multiple herbivore-attacked plants can differ quantitatively or qualitatively compared to single induction (Shiojiri *et al.*, 2001; Rodriguez-Saona *et al.*, 2003; Delphia *et al.*, 2007; De Boer *et al.* 2008; Zhang *et al.*, 2009; Zhang *et al.*, 2013; Erb *et al.* 2010; Schwartzberg *et al.*, 2011). Multiple herbivory can influence indirect plant defense in a positive, neutral, or negative manner. In Lima bean plants, for example, simultaneous feeding of non-prey caterpillars, *Spodoptera exigua* Hübner, and prey, the spider mite *Tetranychus urticae* Koch, results in a synergistic increase in volatile emission and an increased attraction of a natural enemy of the spider mite, i.e. the predatory mite *Phytoseiulus persimilis* Athias-Henriot (De Boer *et al.*, 2008). However, simultaneous feeding of the cicadellid *Euscelidius variegatus* Kirshbaum and *Spodoptera littoralis* Boisduval caterpillars on maize plants did not alter volatile emission differently from *S. littoralis*-induced volatile emission and did not affect behavior of a parasitoid of *S. littoralis* (Erb *et al.*, 2010). Whitefly infestation has a negative effect on predator attraction to *T. urticae*-infested Lima bean plants (Zhang *et al.*, 2009). The underlying mechanism for the differences in the effect of multiple herbivory is likely phytohormone cross-talk (Zhang *et al.*, 2009; Zhang *et al.*, 2013).

Phytohormone crosstalk may also be involved in interactions between herbivores that attack a plant in temporally spaced events (Kessler and Baldwin, 2004; Poelman *et al.*, 2008). Sequential herbivory can have long-lasting effects on plant defenses (Poelman *et al.*, 2008), whereby prior feeding by one herbivore can result in a kind of “vaccination” which can

affect direct and indirect plant defenses against a later-arriving second herbivore (Kessler and Baldwin, 2004). Voelckel and Baldwin (2004) suggest that the order/identity of arrival is crucial because some herbivore-induced stress effects on plant defense seem to be more stable than others. This is likely also dependent on the intensity and timing of subsequent defense inductions. In *Zea mays* seedlings, for example, a low dose of exogenously applied SA increases endogenous JA levels and volatile production upon a second induction by an insect-derived elicitor (Engelberth *et al.*, 2011). However, higher doses resulted in reduced JA responses due to negative cross-talk. Moreover, shorter incubation times than 15 h with the phytohormone did not result in accumulation of JA or enhanced volatile production.

Volatile induction by leaf-chewing lepidopterans, and certain cell-content feeding herbivores, such as the spider mite *Tetranychus urticae*, is primarily regulated by the jasmonic acid pathway, and therefore antagonistic effects on plant defense are not expected between the two (Ozawa *et al.*, 2000). Here, we investigated the effect of sequential induction of plant defense by *M. brassicae* caterpillar oral secretion and an infestation by *T. urticae* spider mites, which were temporally separated by a period of 48 h. We hypothesized that sequential induction of JA-induced plant defenses would result in increased attraction of *P. persimilis* through changes in the HIPV blend. Moreover, we investigated whether transcription levels of the JA-responsive gene *Phaseolus lunatus* β -ocimene synthase (*PIOS*), coding for a rate-limiting step in the biosynthesis of the spider-mite inducible plant volatile (*E*)- β -ocimene, would increase accordingly (Ament *et al.*, 2004). The HIPV (*E*)- β -ocimene is known to be highly attractive to *P. persimilis* (Dicke *et al.*, 1990).

Materials and Methods

Plants

Lima bean plants (*Phaseolus lunatus* L., cv. Wonderbush, De Bruyn Seed Company, Michigan, USA) were cultivated in a greenhouse compartment at 23 ± 2 °C, 60 ± 10 % relative humidity (RH) and a 16 : 8 h light : dark (L : D) photoregime. Plants were grown in 5 × 5 cm plastic pots for gene transcription experiments or 11 × 11 cm for headspace volatile collection and behavioral experiments, respectively. After 12 - 14 days plants with two expanded primary leaves were transferred to a climate chamber and incubated at 25 ± 1 °C, 60 ± 10 % R.H. and 16 : 8 h L : D. In the climate chamber, plants of different treatments were kept separate in plastic cages (90 × 90 × 60 cm) that were connected to house vacuum to prevent volatile transfer between plants of different treatments.

Herbivores and predatory mites

A colony of two-spotted spider mites, *Tetranychus urticae* Koch (Acari: Tetranychidae), was maintained on Lima bean plants in a greenhouse compartment at 25 ± 5 °C, 50 – 70 % R.H., 16 : 8 h L : D. Adult female spider mites for experiments were selected randomly from the colony.

Cabbage moth, *Mamestra brassicae* L. (Lepidoptera: Noctuidae) caterpillars were reared on cabbage plants (*B. oleracea* var. *gemmifera* cv. Cyrus, Syngenta seeds BV, Enkhuizen, The Netherlands) at 22 ± 1 °C and 50 - 70 % R.H., under the same photoregime as for plants and mites. Oral secretions were collected from 18 - 20 caterpillars in the 5th instar, which were randomly selected from the colony.

Predatory mites, *Phytoseiulus persimilis* Athias-Henriot (Acari: Phytoseiidae), were reared in Petri dishes (diameter 9 cm) on detached Lima bean leaves which were heavily infested with *T. urticae* under the same conditions as the *T. urticae* colony. Only gravid female predatory mites were used for behavioral experiments, ca. 1 - 2 days after their final molt. For behavioral experiments the females were individually confined in 0.5 ml Eppendorf tubes containing a piece of moist cotton wool to avoid dehydration. Predatory mites were starved for 24 h and left in the experimental room prior to the experiment in order to acclimate.

Plant treatments

Sequential induction experiments

Plant treatments were: i) control, ii) infestation with 20 spider mites (48 h incubation), iii) caterpillar oral secretion (48 h incubation), iv) caterpillar oral secretion (48 h incubation) followed by infestation with 20 mites (48 h incubation), v) caterpillar oral secretion (96 h incubation). Plants treated with caterpillar oral secretion were first artificially damaged with a pattern wheel by drawing six lines of ca. 7 cm length on each primary leaf. Then the damaged leaves were treated with 10 µl per leaf of diluted caterpillar oral secretion using a fine paint brush for application. Oral secretion consisted of a pooled stock, freshly collected from 18 - 20 5th instar *M. brassicae* caterpillars using a glass Pasteur pipette. The stock was diluted 1:1 with tap water and kept on ice prior to use to avoid degradation of compounds. Upon the application with oral secretion, plants were incubated for 48 h. Control plants and plants receiving single infestation by spider mites did not receive mechanical damage or oral secretion. Plants were kept in a climate chamber and incubated in groups separated by treatment in the cages described above. After 48 h, plants to be analyzed for single induction by oral secretion were sampled for transcriptional, volatile or behavioral analysis. Plants with dual induction treatment or single infestation by *T. urticae* received 20 mites per plant, followed by another 48 h of incubation before sample collection.

Time series experiment

Plant treatments included i) control, ii) infestation with 20 spider mites, and iii) caterpillar oral secretion. Plant treatments were executed the same as in the previous section. Plants were kept in a climate chamber and incubated separately according to treatment in cages. Samples for transcriptional analysis were taken 6, 20, 26, or 46 h following treatment.

Y-tube olfactometer

Responses of predatory mites were tested in a Y-tube olfactometer (Takabayashi & Dicke 1992). A Y-shaped metal wire was located in the center of a glass Y-tube, each arm was connected to a 5-L glass jar. Glass jars containing plants were connected to air inlets providing a 2 L/min charcoal-filtered air influx to carry volatiles into the two arms of the Y-tube. For behavior experiments, plant pots and loose soil were gently removed and roots with soil were carefully wrapped in aluminum foil. Three plants of a treatment were placed in a glass jar as odor source and the system was purged for 30 - 60 min without closing the vessels. Afterwards glass jars were sealed with viton-lined glass lids and the whole Y-tube olfactometer setup was flushed with air for 7 - 10 min before commencing the behavior experiment. Individual predatory mites were placed downwind on the Y-shaped wire and their choice for either odor source recorded when they passed a line located halfway up one of the two olfactometer arms or no-choice was recorded when they had not passed the line within 5 min. Sides of treatments were alternated after every five predatory mites to avoid positional bias. Plants were replaced after every 20 predatory mites or approximately 90 min after the first mite was tested, whichever came first. Each comparison was tested on two different days with 40 - 60 predatory mites per day.

Dynamic headspace collection of plant volatiles

Plants were prepared for volatile collection by gently removing pots and loose soil, and wrapping roots with soil in aluminum foil. Two plants of each treatment were transferred to a 5-L glass jar. Glass jars were sealed with viton-lined glass lids equipped with an air inlet and outlet. The setup was flushed with 100 ml/min synthetic air (Linde Gas Benelux B.V., The Netherlands) filtered by passing through charcoal before entering the glass jar. Glass jars with plant samples were flushed with air for 30 - 45 min. A stainless steel tube filled with 200 mg Tenax TA (20/35 mesh; CAMSCO, Houston, TX, USA) was connected to the outlet of each glass jar and volatile collection was done by sucking air out of the jars at 100 ml/min for 2 h. A total of eight replicates of each treatment were sampled over two days. Fresh weight of above-ground plants tissue was determined immediately after volatile collection using an analytical balance (NewClassic ML, Mettler Toledo, Greifensee, Switzerland).

Chemical analysis of plant volatiles

A Trace Ultra gas chromatograph (GC) coupled with Trace DSQ quadrupole mass spectrometer (MS) both from Thermo (Thermo Fisher Scientific, Waltham, USA) were used for separation and identification of plant volatiles as described previously (Menzel *et al.* 2014).

Standards of (Z)-3-hexen-1-ol, (Z)-3-hexen-1-ol, acetate, (Z)-3-hexen-1-ol, butanoate, (Z)-3-hexen-1-ol, isovalerate, linalool, methyl salicylate, indole, (E)- β -ocimene, as well as alloocimene were obtained from Sigma-Aldrich (Saint Louis, MO, USA). Additional standards (E)-4,8-dimethylnona-1,3,7-triene [(E)-DMNT] and (E,E)-4,8,12-trimethyltrideca-1,3,7,11-tetraene [(E,E)-TMTT] were kindly provided by Prof. W. Boland (Max Planck Institute for Chemical Ecology, Jena, Germany). For quantification, calibration lines were constructed for

each compound using seven data points at different concentrations and two replicates of each data point.

RNA extraction and cDNA synthesis

Spider mites, eggs, feces and webbing were gently removed from plants of the respective treatments using a soft paint brush. Plant material was obtained by cutting four leaf discs out of one primary leaf per plant using a cork borer (diameter 2 cm). Leaf discs obtained from the primary leaf of three plants were pooled to yield one biological replicate. All samples were shock-frozen in liquid nitrogen immediately after collection and then stored at -80 °C until further processing. Frozen leaf material was homogenized using mortar and pestle while avoiding thawing. Total RNA was isolated and purified using the Qiagen (Hilden, Germany) RNeasy Plant Mini kit with integrated DNase treatment, according to manufacturer's instructions. RNA quality was assessed using Agilent 2100 Bioanalyzer with the RNA 6000 Nano Labchip® kit (all from Agilent Technologies, Santa Clara, CA, USA) and RNA quantifications were done using a NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Only RNA samples with 260 / 280 wavelength ratio > 2 and a RIN value > 7 were used for cDNA synthesis. cDNA was generated from total RNA by using the Bio-Rad iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA) according to manufacturer's instructions.

Quantitative RT-PCR

A real-time quantitative RT-PCR was used to quantify gene transcript levels of *P. lunatus* β -ocimene synthase (*PIOS*; GenBank accession EU194553), *acidic pathogenesis-related protein 4* (*PIPR-4*), and the two reference genes *P. lunatus Actin1* (*PIACT1*; GenBank accession DQ159907) and *P. lunatus Nuclear matrix protein 1* (*PINMP1*; GenBank accession AF289260.1). Quantitative RT-PCR was performed in a Rotor-Gene 6000 machine (Corbett Research) with a 72 - well rotor; for a detailed description see Menzel *et al.* (2014). *PIOS* primers were F-*PIOS* 5'-TGCATGGGTCTCAGTCTCTG-3' and R-*PIOS* 5'-TGCTGCTTCCCCTCTCTCTA-3', *PIPR-4* were F-*PIPR-4* 5'-ACGCTTTCCTCAGTGCTCTC-3' and R-*PIPR-4* 5'-TCCTCGTCGTCGCAGTAATCCTT-3', *PIACT1* primers were F-*PIACT1* 5'-CCAAGGCTAACCGTGAAAAG-3' and R-*PIACT1* 5'-AGCCAGATCAAGACGAAGGA-3', and *PINMP1* primers F-*PINMP1* 5'-CCGGAATGGAGTGTGACGAGCA-3' and R-*PINMP1* 5'-CCAGCTCAGAAACATCTGGCAATGG-3'.

Statistical analysis

Log transformation was applied to data from gene transcription experiments and volatiles in order to meet assumptions of normality and homogeneity of variances. Data were analyzed using one-way ANOVA or generalized linear model (GLM) followed by Tukey's Honestly Significant Difference (HSD) post-hoc tests for pairwise comparisons between treatments in the statistical software SPSS version 19 (SPSS Inc., Chicago, IL, USA). Data that violated assumptions on normality and equal variance after log transformation were analyzed by Kruskal-Wallis tests followed by Mann-Whitney U tests applying the Bonferroni correction for multiple comparisons.

Predator choices in the Y-tube olfactometer experiments were analyzed using a binomial test to examine whether the choice distribution significantly differed from 50 : 50.

Volatile profiles of plants exposed to different treatments were analyzed using multivariate data analysis. Data were expressed per unit of plant fresh weight, log-transformed, and univariate-scaled. Then an Orthogonal Projection to Latent Structures-Discriminant Analysis (OPLS-DA) was performed using the software SIMCA P+ version 12 (Umetrics, Umeå, Sweden). Pairwise comparisons for individual volatiles among treatments were executed using Mann-Whitney U tests.

Results

Response of predatory mites to single or multiple herbivore infestation

The attraction of predatory mites to Lima bean plants exposed to different treatments was tested in a two-choice behavioral assay (Fig. 1). Feeding by *T. urticae*, with and without prior treatment with *M. brassicae* oral secretion, were preferred over control plants (binomial test, $P < 0.001$ in both comparisons). However, predators did not distinguish between control plants and plants treated with *M. brassicae* oral secretion alone (binomial test, $P > 0.05$). Moreover, predatory mites showed a preference for odors from plants infested by their prey compared to odors from plants induced by caterpillar oral secretion (binomial test, $P < 0.001$). The volatile blend of plants induced by the combination of non-prey oral secretion and prey herbivores was more attractive than the volatile blend of plants induced by oral secretion of *M. brassicae* (binomial test, $P < 0.001$). Predators did not display a significant preference when they were offered plants infested by prey herbivores versus volatiles from plants induced by the combination of non-prey oral secretion and prey herbivores (binomial test, $P > 0.05$).

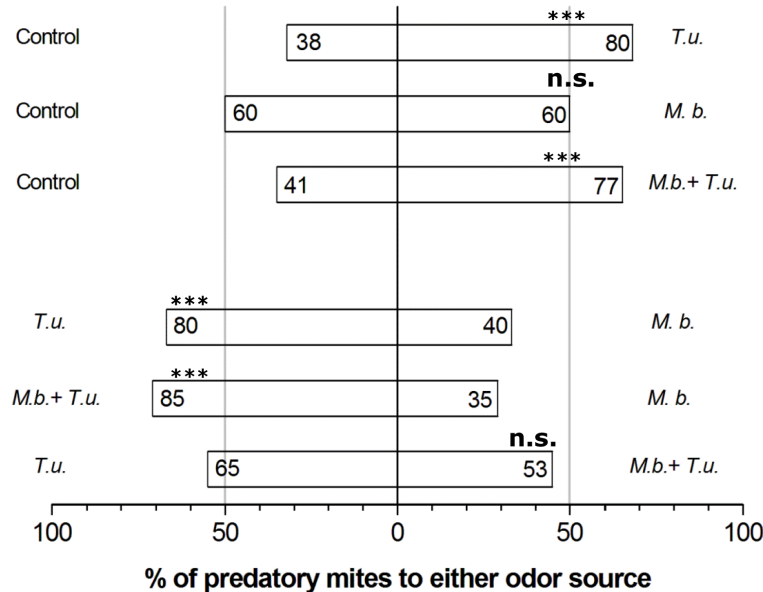


Fig. 1. Responses of *P. persimilis* in a Y-tube olfactometer to volatiles emitted by Lima bean plants induced by mechanical damage and oral secretion of non-prey (*M. brassicae*; *M.b.*), prey (*T. urticae*; *T.u.*) infestation, or a combination of the two (*M.b. + T.u.*). Volatile sources consisted of three Lima bean plants per treatment, i) control plants, or ii) plants infested with 20 *T. urticae* for 48 h, iii) induction by mechanical damage and *M. brassicae* oral secretion incubated for 48 h, or iv) induction by mechanical damage and *M. brassicae* oral secretion incubated for 48 h followed by infestation by 20 *T. urticae* for 48 h. Bars represent the overall percentages of predators choosing either odor source. Numbers in bars correspond to the number of predators choosing either odor source. For each comparison the number of mites that did not make a choice within 5 min ranged between zero and three. Asterisks indicate significance of predatory mite choices (binomial test; n.s. = not significant, *** $P < 0.001$).

Volatile analysis

Spider mite feeding resulted in higher emission rates of several compounds compared to treatment with *M. brassicae* oral secretion (Suppl. Information, Fig. S1 and Fig. S2). Comparison of volatile profiles consisting of the 11 major HIPV compounds emitted in response to the treatments showed that volatile blends were significantly different between treatments: OPLS-DA resulted in a model with three significant principal components (Fig. 2). Figure 2B shows that the first distinction between treatments was made for treatments including exposure to feeding by *T. urticae* versus treatments without *T. urticae*, which were separated by the first principal component. The second component separated treatments with caterpillar oral secretion from treatments that did not include oral secretion. The volatile blend of the combination treatment of caterpillar oral secretion and *T. urticae* was most similar to the volatile blend from plants treated with *T. urticae* infestation alone. Together with the third principal component 89 % of the total variability of the data could be explained. Moreover, the model showed that four volatile compounds, namely (*E,E*)-TMTT, (*E*)- β -ocimene, (*Z*)-3-hexen-1-ol acetate, and alloocimene, had variable importance in the projection (VIP) values higher than 1, thus contributing most to discrimination between treatments. Pairwise comparison between the two treatments (1) 20 *T. urticae* (*T.u.*) and (2) *M. brassicae* oral secretion followed by infestation with 20 *T. urticae* (*M.b.+T.u.*), did not yield significant principal components and thus were not significantly different.

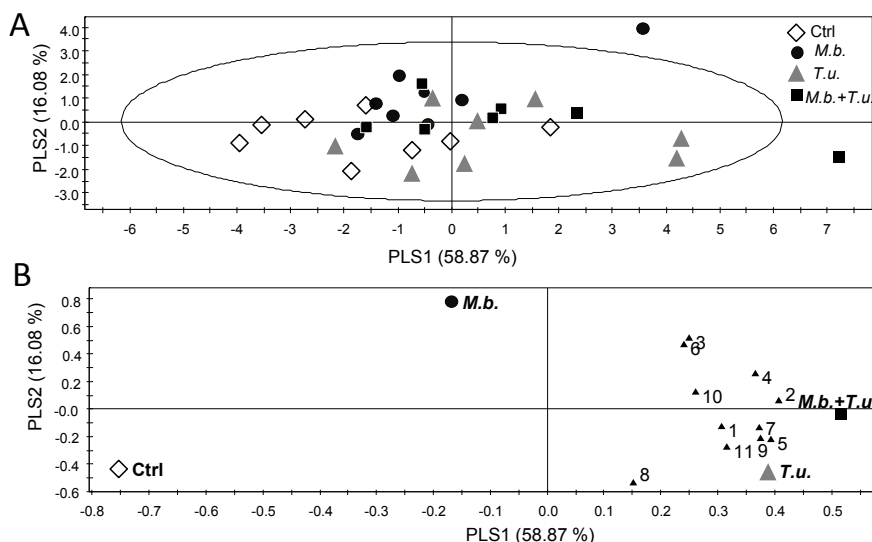


Fig. 2. Multivariate data analysis by orthogonal PLS-DA (OPLS-DA) (panel A) and corresponding loading plot (panel B) of volatile blends of *P. lunatus* plants with i) no treatment (Ctrl), or treated with ii) 20 *T. urticae* for 48 h (*T.u.*), iii) *M. brassicae* oral secretion 48 h (*M.b.*), iv) *M. brassicae* oral secretion 48 h followed by infestation with 20 *T. urticae* for 48 h (*M.b.+T.u.*); eight replicates for each treatment. The first two principal components are depicted (panel A) with the percentage of variation explained in parentheses. Numbers in the loading plot (panel B) represent 1) (*Z*)-3-hexen-1-ol, 2) (*Z*)-3-hexen-1-ol, acetate, 3) (*E*)- β -ocimene 4) linalool, 5) (*E*)-4,8-dimethylnona-1,3,7-triene [(*E*)-DMNT], 6) alloocimene, 7) (*Z*)-3-hexen-1-ol, butanoate 8) methyl salicylate, 9) (*Z*)-3-hexen-1-ol, isovalerate, 10) indole, 11) (*E,E*)-4,8,12-trimethyltrideca-1,3,7,11-tetraene [(*E,E*)-TMTT].

Relative gene transcription

Transcript levels for *PIOS*, the gene encoding for the enzyme mediating the rate-limiting step in the biosynthesis of (*E*)- β -ocimene, which is a principal attractant for *P. persimilis* (Dicke *et al.*, 1990; Zhang *et al.*, 2009), were compared between treatments. Treatments significantly affected *PIOS* transcript levels (GLM, $P < 0.001$; Fig. 3). Plants infested by *T. urticae*, with or without prior treatment with *M. brassicae* oral secretion, showed increased levels of *PIOS* compared to control plants and plants treated with *M. brassicae* oral secretion alone (post hoc Tukey's HSD, $P < 0.01$). Oral secretion from *M. brassicae* did not increase *PIOS* transcript levels different from control (post hoc Tukey's HSD, $P > 0.05$). Transcript levels of *PIPR-4* were also affected by the treatments (ANOVA, $F_{4,15} = 16.86$ $P < 0.001$). Infestation by *T. urticae* and treatment with *M. brassicae* oral secretion both resulted in significantly increased *PIPR-4* transcript levels compared to control plants (post hoc Tukey's HSD, $P < 0.001$). Sequential treatment induced an increase in transcript levels compared to control plants, and transcript levels were even higher than in response to single treatments (post hoc Tukey's HSD, $P < 0.001$).

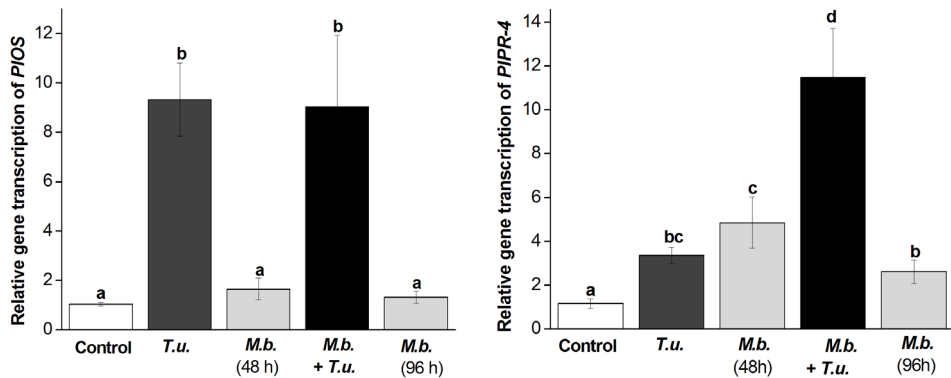


Fig. 3. Relative gene transcript levels of *P. lunatus* β -ocimene synthase (*PIOS*) and *P. lunatus* pathogenesis-related protein 4 (*PIPR-4*) of plants i) without treatment (control), or treated with ii) 20 *T. urticae* for 48 h (*T. u.*), iii) *M. brassicae* oral secretion for 48 h [*M. b.* (48 h)], iv) *M. brassicae* oral secretion for 48 h followed by infestation with 20 *T. urticae* for 48 h (*M. b.* + *T. u.*), v) *M. brassicae* oral secretion 96 h [*M. b.* (96 h)]. Values are the mean (\pm SE) of 10 to 12 biological replicates, pooled from three replications of the same treatment. Different letters above bars indicate significant differences in transcript levels between treatments (Tukey's HSD tests, $P < 0.05$). Gene transcript levels were normalized to the normalization factor obtained from geometrically averaging the Ct values of the two reference genes *P. lunatus* *Actin1* (*PIACT1*) and *P. lunatus* *nuclear matrix protein 1* (*PINMP1*) for each sample.

Time series for relative *PIOS* gene transcription in response to single treatments

To investigate *PIOS* gene transcript levels for plants infested with *T. urticae* and plants with caterpillar oral secretion treatment, a time series experiment was conducted. The time series of *PIOS* gene transcription for the single treatment with 20 *T. urticae* or *M. brassicae* oral secretion showed clear differences in the gene transcription patterns between the two treatments (Fig. 4). Infestation by 20 *T. urticae* led to 14 times higher transcript levels than in control plants already after 6 h post treatment (hpt). At 20 hpt expression levels of both the plants treated with *M. brassicae* oral secretion and plants exposed to *T. urticae* feeding

were significantly higher than control levels (ANOVA, Tukey's HSD tests, $P < 0.05$), whereas at 26 hpt these differences had disappeared. At 46 hpt the same pattern as at 6 hpt was found although the degree of upregulation of *PIOS* was lower. Treatment with caterpillar oral secretion also led to an increase of *PIOS* transcript levels compared to control levels but only at 20 hpt (ANOVA, Tukey's HSD tests, $P < 0.05$). Before and after this time point transcript levels were not different from control levels.

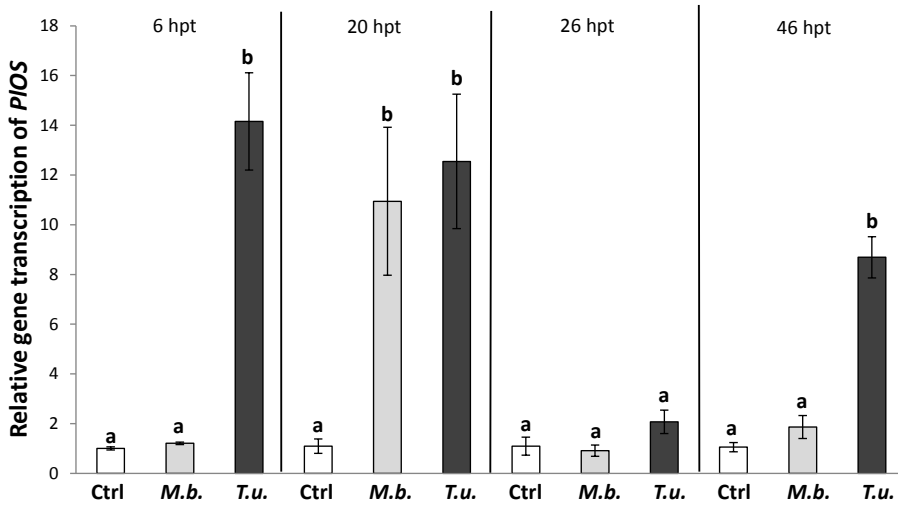


Fig. 4. Relative gene transcript levels of *PIOS* over four time points separated by hours post treatment (hpt). *P. lunatus* plants were treated with i) no treatment (control; Ctrl), ii) 20 *T. urticae* (*T.u.*), or iii) *M. brassicae* oral secretion (*M.b.*). Values are the mean (\pm SE) of four biological replicates. Different letters above bars indicate significant differences in transcript levels between treatments (Tukey's HSD tests, $P < 0.05$) within each time point. Gene transcript levels were normalized to the normalization factor obtained from geometrically averaging the Ct values of the two reference genes *P. lunatus Actin1* (*PIACT1*) and *P. lunatus nuclear matrix protein 1* (*PLNMP1*) for each sample.

Discussion

Plants are frequently attacked by multiple herbivores, which may arrive at different moments in time. The resulting sequential herbivory has been shown to have long-lasting effects on plant resistance against the subsequent herbivores (Viswanathan *et al.*, 2007; Poelman *et al.*, 2008; Mathur *et al.*, 2011; Stam *et al.*, 2014). However, little is known about the effect of sequential herbivory on plant indirect defense (but see e.g. Zhang *et al.*, 2009; Erb *et al.*, 2010; Zhang *et al.*, 2013). Here, we investigated the effect of the sequential attack by different herbivore species on plant indirect defense against *T. urticae* spider mites through the attraction of the specialist predator *P. persimilis*. Previous studies suggest that plants can form memories after stressful events such as herbivory, which enables them to adjust their defense accordingly in order to respond in an enhanced manner to a second stress (Frost *et al.*, 2008; Conrath, 2009). Our results show that prior treatment of plants with oral secretions of the generalist caterpillar *M. brassicae*, as a mimic of caterpillar feeding, does not affect the attraction of *P. persimilis* to plants infested with its prey *T. urticae*. The modulating effects caused by interactions with two herbivore species may thus depend on several factors such as severity of initial damage or infestation, timing between attacks, identity of the herbivore, and the associated defense pathway induced (Voelckel and Baldwin, 2004; Viswanathan *et al.*, 2007; Dicke *et al.*, 2009; Zhang *et al.*, 2009). The defense pathway commonly induced by leaf-chewing insects such as *M. brassicae*, is the octadecanoid pathway with JA as signaling molecule (McConn *et al.*, 1997; Kessler and Baldwin, 2002). The same defense pathway is also induced by feeding by cell-content feeders such as *T. urticae* (Dicke *et al.*, 1999; Ozawa *et al.*, 2000; Li *et al.*, 2002). Multiple herbivory that occurs simultaneously by herbivores that induce the same pathway may lead to changes in volatile emission that results in increased attraction of predators (De Boer *et al.*, 2008). In our study with sequential herbivory, predators did not distinguish between volatile blends from plants fed upon by *T. urticae* only and plants pre-treated with caterpillar oral secretions and subsequently exposed to feeding by *T. urticae*. Headspace analysis demonstrated that the volatile profiles of plants that had been exposed to these two treatments largely overlapped. It is thus possible that the initial defense induction was not strong enough to induce a memory effect or that the memory had decayed at the onset of the second attack by *T. urticae*. However, the volatile profile from plants with caterpillar oral secretion treatment was notably different compared to plants treated with *T. urticae* and predators were significantly more attracted to plants infested with their prey. This is in accordance with data of De Boer and co-workers (2008) who found that the specialist predator *P. persimilis* can distinguish between volatiles induced by prey and non-prey herbivores. Nevertheless, De Boer *et al.* (2008) found an increased attraction of *P. persimilis* to dual-infested Lima bean plants, which were fed upon by *T. urticae* and *Spodoptera exigua* caterpillars. In their study the dual infestation resulted in the emission of increased amounts of a subset of the plant volatiles. In the study by De Boer *et al.* (2008), the plants were exposed to simultaneous infestation with spider mites and *S. exigua* caterpillars. Whether the differentiation by the predators in their study and not in ours was due to the different caterpillar species, simultaneous compared to sequential treatments, or to caterpillar feeding instead of the use of oral secretion, remains to be elucidated.

It has been shown that *P. persimilis* is attracted to five HIPVs, two of which, namely (*E,E*)-TMTT and (*E*)- β -ocimene, were indeed found to play a significant role in separating the volatile profiles of Lima bean plants that had received different treatments (Dicke *et al.*, 1990; De Boer and Dicke, 2004). Dicke *et al.* (1999) found that the emission rates of the two homoterpenes (*E,E*)-TMTT and (*E*)-DMNT, as well as the phenolic ester MeSA, were involved in the differentiation between JA-treated plants and *T. urticae*-treated plants, the latter being more attractive to *P. persimilis*. The two other compounds, namely the terpene alcohol linalool and the monoterpene (*E*)- β -ocimene are also known to play important roles in the attraction of *P. persimilis* to *T. urticae*-infested Lima bean plants (Dicke *et al.*, 1990; Zhang *et al.*, 2009). Arimura *et al.* (2008) found a close relationship between JA levels and transcription of *PIOS*, which is the enzyme that leads to the production of the principal predator attractant (*E*)- β -ocimene in Lima bean. Voelckel and Baldwin (2004) found that simultaneous and sequential herbivory can cause different patterns of gene transcription when compared to individual feeding by the two different herbivores. In our study, treatments with the prey *T. urticae* and with the prey plus *M. brassicae* oral secretion resulted in increased *PIOS* transcript levels in Lima bean plants, but not different from each other. Treatment with caterpillar oral secretion, which did not result in attraction of *P. persimilis*, showed *PIOS* transcript levels comparable to control levels. However, De Boer *et al.* (2008) found that Lima bean plants infested with *S. exigua* caterpillars or *T. urticae* spider mites do both emit large amounts of (*E*)- β -ocimene. Moreover, it is likely that a time lag exists between gene transcription and metabolite production (Stam *et al.*, 2014). To investigate the temporal effect of caterpillar oral secretion on *PIOS* transcript levels a time series experiment was conducted. Results show that treatment with caterpillar oral secretion does increase *PIOS* transcript levels. Moreover, *PIOS* transcript levels of plants treated with caterpillar oral secretion peaked at a different time point than in plants infested with *T. urticae* infestation and showed different transcriptional patterns. Compared to *T. urticae*-treated plants, *PIOS* transcription peaked quite late for plants treated with caterpillar oral secretion. This is in accordance with Arimura *et al.* (2008) who found that feeding by the generalist caterpillar *S. littoralis* induced *PIOS* transcript levels only after 24 hpt but not at 6 hpt, whereas JA treatment and wounding did already induce *PIOS* after 6 h. Continuous infestation and thus feeding by *T. urticae* resulted in elevation of *PIOS* transcript levels in three out of four time points. Moreover, *PIOS* transcript levels of plants treated with caterpillar oral secretion declined to control levels within 6 h after the peak. Apart from an effect of *M. brassicae* oral secretion on transcription of *PIOS*, also transcription of another gene investigated was affected. Transcription levels of *PIPR-4* were upregulated by *T. urticae* feeding as well as application of *M. brassicae* oral secretion, and combined application led to higher transcript levels than either single treatment. *PIPR-4* is an acidic chitinase, which is MeSA-responsive (Margis-Pinheiro *et al.*, 1991; Arimura *et al.*, 2000). Chitinases are commonly involved in plant direct defense against plant pathogens and arthropods (Schlumbaum *et al.*, 1986; Kramer and Muthukrishnan, 1997). Consequently, other defense mechanisms apart from indirect defense could in fact be affected by the combination treatment of *T. urticae* and *M. brassicae* oral secretion.

In our study, predatory mite behavior was well reflected in volatile blend analysis. Four compounds, i.e. (*E,E*)-TMTT, (*E*)- β -ocimene, (*Z*)-3-hexen-1-ol acetate, and alloocimene, were

found to have an important function in discrimination between treatments. The involvement of (*E,E*)-TMTT and (*E*)- β -ocimene in the attraction of *P. persimilis* to prey-infested Lima bean plants have been described previously (Dicke *et al.*, 1990). Nevertheless, there are other well-known compounds that play an important role in predator attraction (Dicke *et al.*, 1990; De Boer *et al.*, 2004). In fact, volatile blends convey more information than a single compound e.g. on herbivore identity and herbivore developmental stage (Takabayashi *et al.*, 1995; De Moraes *et al.*, 1998; Mumm and Dicke, 2010).

Conclusion

In our study, sequential herbivore treatment involving herbivores that induce the same plant defense pathway did not enhance or interfere with indirect defense against *T. urticae*. Yet, differences in volatile blends perceived by the predator play an important role in distinguishing between prey and non-prey infested plants. Volatiles attractive to natural enemies are perceived in the context of other volatiles in order to extract specific information about the presence of prey. Furthermore, gene transcription is differently induced in terms of timing and magnitude by different herbivore species and a considerable time lag exists between gene transcription of genes relevant in indirect defense and metabolite emission.

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Chapter 6

Effect of sequential herbivory by conspecific herbivorous spider mites on gene transcription and volatile emission in Lima bean plants

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Abstract

Plants possess a manifold of defense mechanisms that protect them against herbivorous arthropods in nature. However, due to their sessile nature plants may frequently encounter attacks of the same species of herbivores. It can be expected that plants have evolved defense mechanisms which help them in recognizing and defending themselves more efficiently against re-occurring attacks. Here, we studied the effect of herbivory by two-spotted spider mites (*Tetranychus urticae*) on two components of Lima bean induced indirect defense against subsequent herbivory by conspecifics. We studied the emission of (*E*)- β -ocimene and 12 other plant volatiles commonly associated with herbivory, as well as the transcription of two genes involved in Lima bean defense, namely *P. lunatus* β -ocimene synthase (*PIOS*) and *P. lunatus* pathogenesis-related protein 4 (*PIPR-4*). Volatile profiles and gene transcript level of plants attacked by herbivores differed significantly from that of control plants. Emission of volatiles did not differ between plants that experienced two bouts of herbivore attack by conspecific spider mites compared to plants that experienced only one bout of spider mite attack. Moreover, transcript levels of *PIOS* and *PIPR-4* did not differ for these treatments. Our results suggest that Lima bean plants do not increase defense in response to sequential herbivory by two-spotted spider mites under the exposure regime tested.

Introduction

Plants are sessile organisms without chance of evading attacks by herbivorous arthropods. It has been suggested that plants are able to form some sort of 'memory' in response to a herbivore or microbial challenge. This memory formation enables plants to respond in a faster and stronger manner to a subsequent attack, which is also known as "priming" (Frost *et al.*, 2008; Conrath, 2009). In fact, plants defend themselves against herbivorous arthropods by utilization of an arsenal of sophisticated defense mechanisms. These defense mechanisms can be roughly divided into direct defense mechanisms, such as thorns and toxic compounds, and indirect defense mechanisms, which involve the attraction and employment of natural enemies of herbivores (Schoonhoven *et al.*, 2005). Moreover, a distinction is made between constitutive and induced defense mechanisms. The latter, induced defenses, are highly plastic, and enable plants to respond in a highly specific manner to an attacker (Agrawal, 2001). Moreover, initial herbivore-induced changes in plant chemistry may occur within hours, and the effects may persist for hours to weeks, thereby affecting defense against other herbivores (Stam *et al.*, 2014).

Carnivorous enemies of herbivores use a number of chemical cues to locate and identify their herbivorous prey (Mumm and Dicke, 2010). Plants release a number of volatile organic compounds, known as herbivore-induced plant volatiles (HIPVs), which are produced *de novo* or are incrementally released and play a crucial role in attracting natural enemies of a specific herbivore (Paré and Tumlinson, 1997; Dicke and Baldwin, 2010). Extensive research has been carried out to study simple tritrophic interactions between a plant, an herbivore, and its natural enemy. HIPV blends are complex mixtures consisting of 20 to 200 compounds, which can provide a manifold of information depending on quantitative and qualitative differences in the blend composition (Dudareva *et al.*, 2004). Consequently, plants do not only provide information on the species of the attacking herbivore to carnivores (De Moraes *et al.*, 1998; Turlings *et al.*, 1998), but also more detailed information such as on herbivore developmental stage (Takabayashi *et al.*, 1995), and herbivore density (Gols *et al.*, 2003).

Nevertheless, plants are freely accessible to herbivores in nature, and plants are likely to experience events of multiple herbivory, which can directly or indirectly influence tritrophic interactions (Dicke *et al.*, 2009). However, only few studies have investigated the modifying effects of multiple herbivores on tritrophic interactions (Dicke *et al.*, 2009). Some of the available studies suggest that natural enemies tend to prefer volatiles from plants infested with multiple herbivore species over volatiles from single infestation by either of the herbivore species (Moayeri *et al.* 2007; De Boer *et al.* 2008). Other studies suggest no impact or a negative impact of multiple herbivory on plant defense (Zhang *et al.*, 2009; Erb *et al.*, 2010). These differences in the effect on defense manifestation are likely caused by differential induction and antagonistic effects among phytohormones, such as jasmonic acid (JA) and salicylic acid (SA), that are crucial in plant defense induction (Pieterse *et al.*, 2012; Stam *et al.*, 2014).

Depending on herbivore species and feeding mode, the JA defense signaling pathway or the SA signaling pathway can be induced. Each of them activates a set of genes which are involved in generating a distinct plant defense response (Pieterse *et al.*, 2012). Herbivory by arthropods with the same feeding mode or induction of the same defense signaling pathway are therefore expected to cause no interference with defense responses within the plant. In fact, De Boer *et al.* (2008) showed that simultaneous feeding of the beet armyworm *Spodoptera exigua* Hübner potentiated indirect defense against the spider mite *Tetranychus urticae* Koch. Defense against biting-chewing herbivores and certain cell-content feeding mites involves for example JA-related defenses (Ozawa *et al.*, 2000; Pieterse *et al.*, 2012). Nevertheless, De Vos *et al.* (2005) found that herbivores with different feeding modes induce distinctive transcriptional patterns in Arabidopsis. In fact, induced defense responses against these two groups of herbivores vary enough to allow predators a distinction between HIPV induced by a prey and a non-prey herbivore (De Boer *et al.*, 2008).

Generally, feeding by conspecifics can increase plant resistance and HIPVs from plants fed upon by herbivores may even repel conspecific herbivores and render neighboring plants more resistant to attack by the same herbivore (Dicke, 1986; Karban, 1990; Bruin *et al.*, 1992; De Moraes *et al.*, 2001; Horiuchi *et al.*, 2003). Moreover, Cui *et al.* (2012) found that feeding by conspecifics significantly reduced whitefly fitness. However, also effects induced by conspecifics may not always be of positive nature for plants. For instance, Underwood *et al.* (2012) found that single damage by the generalist caterpillar *Spodoptera exigua* induced plant defense, but repeated damage by the caterpillar interfered with plant defense.

Here we investigated the effect of a low-density infestation by the spider mite *T. urticae*, on the induction of defenses by conspecifics at a later time point; we focused on the induction of HIPV and genes involved in induced defense. We hypothesized that a defense induction by conspecific herbivores would result in an enhanced induced defense response against the later-arriving conspecific herbivores.

Methods and materials

Plants and mites

Lima bean plants (*Phaseolus lunatus* L., cv. Wonder Bush) were maintained in a greenhouse at 23 ± 2 °C with 60 ± 10 % R.H., and a photophase of 16 h. Plants were used for experiments 9 - 10 days after sowing. Two-spotted spider mites, *Tetranychus urticae* Koch (Acari: Tetranychidae), were reared on Lima bean plants in a greenhouse compartment at 25 ± 5 °C, R.H. 50 – 70%, 16L : 8D. Only adult females were used for the infestation of the plants in experiments.

Plant treatments

Plant treatments consisted of i) control, ii) four *T. urticae*, iii) four *T. urticae* followed by no treatment, iv) four *T. urticae* followed by no treatment and then 10 *T. urticae*, and v) 10 *T. urticae* (see also Suppl. Fig. S1 - 2). Control plants [i)] did not receive any treatment. The

two primary leaves of plants with treatment ii) received two adult *T. urticae* females per leaf that were transferred from a plant in the spider-mite culture by using a fine paint brush. After 48 h mites and their residues were removed by using a fine paint brush and leaf tissue was sampled. Plants of treatment iii) received the same treatment as for ii), except that they were incubated for another 48 h after removal of mites and their residues. Primary leaves of plants with treatment iv) were also infested with two adult *T. urticae* females per primary leaf for 48 h. After 48 h, the mites and their residues were removed, and plants were incubated for another 48 h. Subsequently, the primary leaves were infested with five adult *T. urticae* per leaf. After another 48 h, the mites and their residues were removed and plant leaf tissue was sampled. For treatment v) at 96 h after start of experiments plants were infested with five adult *T. urticae* per primary leaf and were incubated for 48 h before sampling. Each treatment was applied to 6 - 15 plants, except the control treatment for which the number of plants was 48 - 60.

RNA extraction and cDNA synthesis

Plant material was collected and processed as previously described in Menzel *et al.* (2014). Plant material from primary leaves of three plants was pooled to give one biological replicate and three to five biological replicates were collected for each treatment per experimental replication.

Quantitative RT-PCR

In order to assess relative transcript levels of the genes of interest, a real time quantitative RT-PCR was used. Relative transcript levels of *P. lunatus* β -ocimene synthase (*PIOS*; GenBank accession EU194553), *P. lunatus* acidic pathogenesis-related protein 4 (*PIPR-4*), and the two reference genes *P. lunatus* Actin1 (*PIACT1*; GenBank accession DQ159907) and *P. lunatus* Nuclear matrix protein 1 (*PINMP1*; GenBank accession AF289260.1) were quantified. Quantitative RT-PCR was performed in a Rotor-Gene 6000 machine (Corbett Research) with a 72-well rotor; for a detailed description see Menzel *et al.* (2014). *PIOS* primers were F-*PIOS* 5'-TGCATGGGTCTCAGTCTCTG-3' and R-*PIOS* 5'-TGCTGCTTCCCCTCTCTCTA-3', *PIPR-4* were F-*PIPR-4* 5'-ACGCTTTCCTCAGTGCTCTC-3' and R-*PIPR-4* 5'-TCCTCGTCGTCGCAGTAATCCTT-3', *PIACT1* primers were F-*PIACT1* 5'-CCAAGGCTAACCGTGAAAAG-3' and R-*PIACT1* 5'-AGCCAGATCAAGACGAAGGA-3', and *PINMP1* primers F-*PINMP1* 5'-CCGGAATGGAGTGTTGACGAGCA-3' and R-*PINMP1* 5'-CCAGCTCAGAAACATCTGGCAATGG-3'.

Dynamic headspace collection of plant volatiles

For volatile collection, samples were taken from plant treatments i), and iii)-v) (see Suppl. Fig. S2). Plants were prepared for volatile collection by carefully wrapping their pots containing soil and roots with aluminum foil, thereby leaving only the above-ground part of the plants uncovered. Then four plants of each treatment were transferred to a 30-L glass jar. Glass jars were sealed with viton-lined glass lids equipped with an air inlet and outlet. The setup was flushed with 200 ml/min synthetic air (Linde Gas Benelux B.V., The Netherlands), which was filtered by passing through charcoal before entering the glass jar. Glass jars with plants were

flushed with air for 45-60 min before sampling. For sampling a stainless steel tube filled with 200 mg Tenax TA (20/35 mesh; CAMSCO, Houston, TX, USA) was connected to the outlet of each glass jar and volatiles were collected by sucking air out of the jars at 200 ml/min for 2 h. A total of 10 replicates of each treatment were sampled over 10 days. Immediately after volatile collection, the fresh weight of above-ground tissue of sampled plants was determined using an analytical balance (NewClassic ML, Mettler Toledo, Greifensee, Switzerland).

Chemical analysis of plant volatiles

Separation and identification of plant volatiles was done using a Trace Ultra gas chromatograph (GC) coupled with Trace DSQ quadrupole mass spectrometer (MS) (Thermo Fisher Scientific, Waltham, USA). For a detailed description we refer to Menzel *et al.* (2014).

Standards of (*E*)-2-hexenal, (*Z*)-3-hexen-1-ol, (*Z*)-3-hexen-1-ol, acetate, (*Z*)-3-hexen-1-ol, butanoate, (*Z*)-3-hexen-1-ol, isovalerate, linalool, methyl salicylate, indole, β -caryophyllene, (*E*)- β -ocimene, and alloocimene were acquired from Sigma-Aldrich (Saint Louis, MO, USA). Additional standards (*E*)-4,8-dimethylnona-1,3,7-triene [(*E*)-DMNT] and (*E,E*)-4,8,12-trimethyltrideca-1,3,7,11-tetraene [(*E,E*)-TMTT] were kindly provided by Prof. W. Boland (Max Planck Institute for Chemical Ecology, Jena, Germany). For quantification, calibration lines were constructed for each compound using seven data points at different concentrations and two replicates for each data point.

Statistical analysis

Normally distributed $2^{-\Delta\Delta Ct}$ values for gene transcript levels of *PIOS* and *PIPR-4* with homogeneity of variances were analyzed by Student's t-test for pairwise comparisons or one-way ANOVA followed by Tukey's Honestly Significant Difference (HSD) for post hoc pairwise comparisons. Gene transcription data of *PIPR-4* had to be log-transformed prior to analysis by one-way ANOVA in order to meet the assumptions. Gene transcription data that violated assumptions of normality and homogeneity of variances were analyzed with Mann-Whitney tests for pairwise comparisons. Statistical analyses were performed in SPSS version 19 (SPSS Inc., Chicago, IL, USA). Volatile profiles of plants exposed to different treatments were analyzed using multivariate data analysis. Data were expressed as amount emitted per hour per unit of plant fresh weight, log-transformed, and univariate-scaled. Then a Latent Structures-Discriminant Analysis (PLS-DA) was performed using the software SIMCA P+ version 12 (Umetrics, Umeå, Sweden).

Results

Volatile emission

Emission profiles of plants with different treatments were compared for 13 well-known HIPVs emitted by spider-mite infested Lima bean plants (Fig. 1). PLS-DA with all four treatments resulted in a model with one significant principal component that explained 50 % of the total variability of the data. Volatile profiles of control plants were clearly different from plants with

the different treatments (see also pairwise comparisons Suppl. Fig. S3 - 5). Three volatile compounds, namely (Z)-3-hexen-1-ol, acetate, β -caryophyllene, and indole had variable importance in the projection (VIP) values higher than 1 in the model, meaning that those volatiles contributed most to discrimination between treatments. Volatile profiles of plants with 10 *T. urticae* with and without previous infestation by conspecifics overlapped largely and were closely located to each other (Fig. 1 A & B). Moreover, pairwise comparisons by OPLS-DA did not result in a significant model. For the results of single volatile analyses between different treatments refer to supplemental data Table S1.

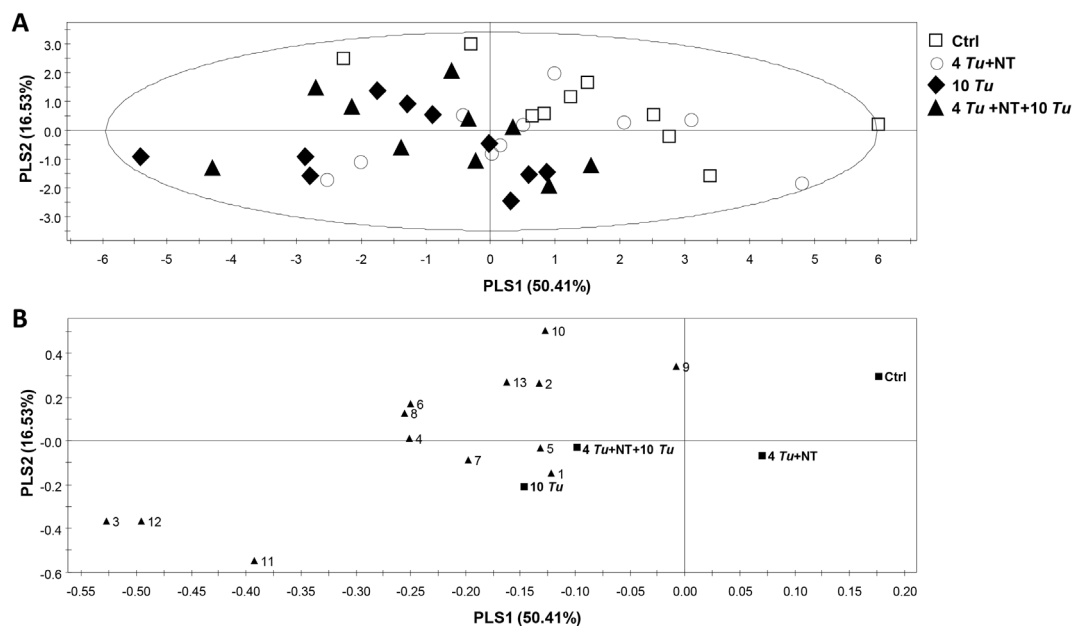


Figure 1. Multivariate data analysis by PLS-DA (panel A) and corresponding loading plot (panel B) of volatile blends of *P. lunatus* plants with i) no treatment (Ctrl), or treatment with ii) four *T. urticae* for 48 h followed by no treatment for 48 h (4 *Tu*+NT), iii) four *T. urticae* for 48 h followed by no treatment for 48 h and subsequent infestation 10 *T. urticae* for 48 h (4 *Tu*+NT+10 *Tu*), iv) no treatment for 96 h and 10 *T. urticae* for 48 h (10 *Tu*). The first two principal components are given (panel A) with the percentage of variation explained in parentheses. Numbers in the loading plot (panel B) represent 1) (*E*)-2-hexenal, 2) (Z)-3-hexen-1-ol, 3) (Z)-3-hexen-1-ol, acetate, 4) (*E*)- β -ocimene, 5) linalool, 6) (*E*)-4,8-dimethylnona-1,3,7-triene [(*E*)-DMNT], 7) alloocimene, 8) (Z)-3-hexen-1-ol, butanoate, 9) methyl salicylate, 10) (Z)-3-hexen-1-ol, isovalerate, 11) indole, 12) β -caryophyllene, and 13) (*E,E*)-4,8,12-trimethyltrideca-1,3,7,11-tetraene [(*E,E*)-TMTT].

Relative gene transcript levels of *PIOS* and *PIPR-4*

Relative transcript levels of *PIOS*, the gene which codes for the enzyme ocimene synthase, which is involved in the synthesis of the volatile compound (*E*)- β -ocimene, were compared among treatments (Fig. 2). Transcript levels of *PIOS* were significantly increased in plants that were infested by four *T. urticae* for 48 h compared to transcript levels in control plants without infestation (Mann-Whitney U test, $P = 0.001$). However, after *T. urticae* had been removed, *PIOS* transcript levels returned to control levels and were not significantly different from control

levels after 48 h (Student's t-test, $P = 0.43$). When plants were infested with 10 *T. urticae*, *PIOS* transcript levels were significantly affected (ANOVA $F_{2,17} = 4.40$, $P < 0.05$). Nevertheless, *PIOS* transcript levels in plants that had previously experienced an infestation by conspecifics were not significantly different from transcript levels of plants that had only experienced a 48 h period of spider-mite infestation (Tukey's post hoc test, $P > 0.05$). Moreover, *PIOS* transcript levels of plants with infestation experience by conspecifics were not different from control levels (Tukey's post hoc test, $P > 0.05$).

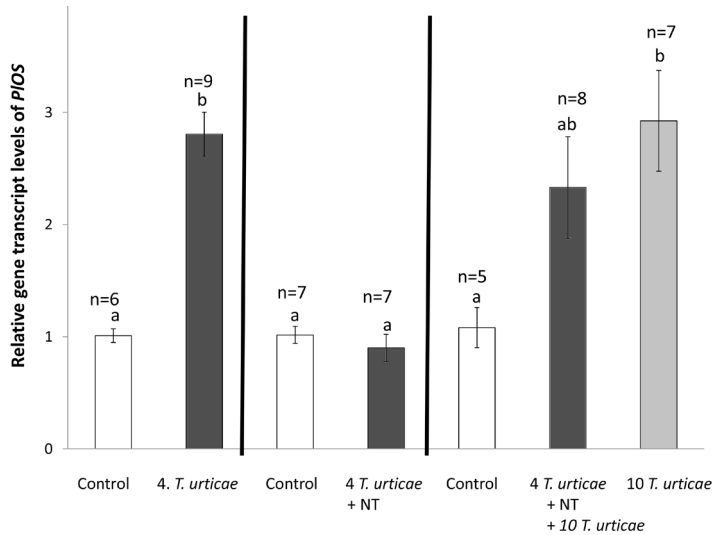


Figure 2. Relative gene transcript levels of *PIOS* in Lima bean plants. Plant were i) control, or treated with ii) four *T. urticae* for 48 h, iii) four *T. urticae* 48 h followed by no treatment for 48 h, iv) four *T. urticae* for 48 h followed by no treatment for 48 h and then 10 *T. urticae* for 48 h, v) 10 *T. urticae* for 48 h. Values are the mean (\pm SE) of five to nine biological replicates, pooled from two replications of the same treatment. Different letters above bars indicate significant differences in transcript levels between treatments (Mann-Whitney U test, Student's t-test, and Tukey's HSD tests respectively, $P < 0.05$). Gene transcript levels were normalized to the normalization factor obtained from geometrically averaging the Ct values of the two reference genes *P. lunatus Actin1* (*PIACT1*) and *P. lunatus nuclear matrix protein 1* (*PINMP1*) for each sample.

Relative gene transcript levels of *PIPR-4*, a chitinase which may be involved in plant direct defense were compared among treatments (Ward *et al.*, 1991; Arimura *et al.*, 2000). Gene transcript levels of *PIPR-4* were significantly increased compared to control levels when plants were infested with four *T. urticae* (Fig. 2; Mann-Whitney U test, $P = 0.001$). After four *T. urticae* had been removed and plants had recovered for 48 h, *PIPR-4* transcript levels were not different from control levels (Student's t-test, $P = 0.20$). When plants were infested with 10 *T. urticae*, treatment significantly affected *PIPR-4* transcript levels compared to control plants (ANOVA $F_{2,17} = 14.28$, $P < 0.001$). Plants that were infested with 10 *T. urticae* and plants that had also previously experienced an infestation by conspecifics showed increased *PIPR-4* levels compared to control plants (Tukey's post hoc both, $P < 0.01$), while they were not different from each other (Tukey's post hoc both, $P < 0.05$).

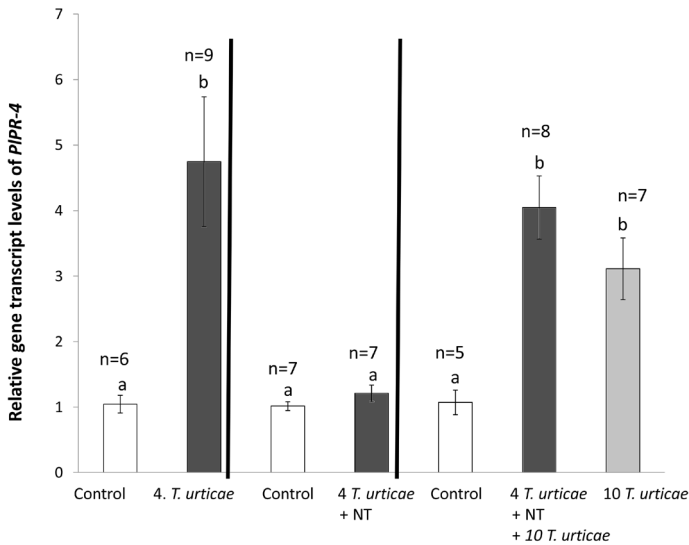


Figure 3. Relative gene transcript levels of *PIPR-4* in Lima bean plants. Plants were i) control, or treated with ii) four *T. urticae* for 48 h, iii) four *T. urticae* for 48 h followed by no treatment for 48 h and then 10 *T. urticae* for 48 h iv) four *T. urticae* for 48 h followed by no treatment for 48 h and then 10 *T. urticae* for 48 h v) 10 *T. urticae* for 48 h. Values are the mean (\pm SE) of five to nine biological replicates, pooled from two replications of the same treatment. Different letters above bars indicate significant differences in transcript levels between treatments (Mann-Whitney U test, Student's t-test, and Tukey's HSD tests respectively, $P < 0.05$). Gene transcript levels were normalized to the normalization factor obtained from geometrically averaging the Ct values of the two reference genes *P. lunatus Actin1* (*PLACT1*) and *P. lunatus nuclear matrix protein 1* (*PLNMP1*) for each sample.

Discussion

Induced defense responses of plants involve a time-lag between recognition of attack by herbivores and activation of defenses. In order to reduce this time lag between attacker recognition and defense induction, it might be advantageous for plants to retain information by memory formation about a previous herbivore attack. In this study, we investigated whether a low density infestation by the spider mite *T. urticae* could affect the emission of 13 relevant HIPVs and two genes involved in induced plant defense, when plants experienced a second bout of defense induction by conspecifics at a later time point. Our results show that volatile profiles and transcript levels of genes which function in Lima bean defense, *PIOS* and *PIPR-4*, did not differ for plants infested with *T. urticae*, irrespective of their previous encounter with the same herbivore.

Indirect plant defense by natural enemies of herbivores relies on the emission of HIPVs as reliable host location cues. The tritrophic interaction of Lima bean plants with the specialist predator mite *Phytoseiulus persimilis* Athias-Henriot, an important natural enemy of spider mites, has been extensively studied and the principal volatile compounds that mediate the tritrophic interaction have been identified (Dicke *et al.*, 1990; De Boer and Dicke, 2004). We have previously found that indirect defense against *T. urticae* mediated by *P. persimilis* can be enhanced when plants are treated with exogenous JA for up to 7 days after defense induction (Gols *et al.*, 2003; Menzel *et al.*, 2014). Nevertheless, in the present study we found no effect on indirect defense for sequential infestation by conspecifics in terms of volatile emission. The acyclic monoterpene hydrocarbon compound (*E*)- β -ocimene constitutes one of the most common volatile chemicals released from plants in response to herbivory and is a principle attractant for the specialist predatory mite *P. persimilis* (Dicke *et al.*, 1990; Paré and Tumlinson, 1999; Pichersky and Gershenzon, 2002; Arimura *et al.*, 2009). The corresponding gene, *β -ocimene synthase* is also known as *PIOS* in Lima bean and codes for the enzyme which is involved the last step of the synthesis of this compound (Ament *et al.*, 2004; Arimura *et al.*, 2004). Arimura *et al.* (2008) showed that *PIOS* is a JA-regulated gene that responds to increases in JA levels with increased transcript accumulation. Infestation by *T. urticae* mites has previously been shown to induce JA and *de novo* synthesis of HIPVs, such as (*E*)- β -ocimene (Dicke *et al.*, 1990; Ozawa *et al.*, 2000). Accordingly, we found that an infestation by four *T. urticae*, and later 10 *T. urticae*, increased transcript levels of *PIOS* compared to control transcript levels. However, after removal of *T. urticae* gene transcript levels returned to control levels within 48 h. In fact, plant induced defenses, such as the activation of defense genes and synthesis of HIPVs, are considered as costly in the absence of herbivores due to a metabolic trade-off between plant growth and reproduction with these defenses (Heil and Baldwin, 2002; Kessler and Baldwin, 2002). Consequently, induced defenses are usually quickly down-regulated in the absence of attackers. However, “priming” of defenses against subsequent herbivores is thought to outweigh the costs for this type of defense (Van Hulten *et al.*, 2006). Nevertheless, in accordance with volatile results we did not find an effect on *PIOS* gene transcription. Moreover, direct defense mediated by chitinases such as *PR-4* seemed to follow the same transcriptional pattern as for *PIOS*, not indicating a memory effect. Previously, Underwood (1998) showed that soybean plants became more resistant

to Mexican bean beetles by 3 days after attack by conspecifics. Moreover, Viswanathan *et al.* (2007) found season-long effects of sequential flea beetle feeding in *Solanum dulcamara* L. However, factors such as infestation levels and severity of damage level, and time interval between inductions can have a significant impact on induced plant defense responses (e.g. Underwood, 1998; Gols *et al.*, 2003). The two spotted-spider mite *T. urticae* is a cell-content feeder, which causes small lesions in plant tissues. Large numbers of these herbivorous mites are known to overexploit their host plants, but low numbers of *T. urticae*, such as the four used for the first infestation in our experiments, cause little plant tissue damage compared to other herbivores. Consequently, initial infestation might have been too low or lasted too short in order to generate a defense response potent enough to induce memory formation. Moreover, while JA is a key regulator of defense against *T. urticae*, it has been shown that defense against these mites also induces SA (Ozawa *et al.*, 2000; Ament *et al.*, 2004). The antagonistic cross-talk between the JA and SA signaling pathway might thus pose an obstacle in the formation of defense memory against *T. urticae* infestation.

Conclusion

Conspecific herbivore infestation by *T. urticae* does not enhance indirect plant defense in terms of a memory effect for volatile emissions or defense gene transcription. Absence of a priming effect might be caused by antagonistic cross-talk of phytohormone signaling pathways or low herbivore density.

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Chapter 7

General discussion

Tila R. Menzel

Introduction

Plants live in complex environments which require them to interact with a manifold of arthropods, including insects, of which ca. 50% are herbivores. However, interactions with arthropods can also benefit plants, in particular interactions with natural enemies of herbivores or even be vital for plant reproduction through interactions with pollinators. Chemical cues, such as plant volatiles, play an important role in the interactions of plants with their surrounding community (Dicke and Van Loon, 2000). Volatiles can be used by herbivores to locate their respective host plants or repel herbivores from a potential host plant (Bolter *et al.*, 1997; Arimura *et al.*, 2000; Kalberer *et al.*, 2001; Conrath, 2009). Moreover, herbivore-induced plant volatiles (HIPVs) emitted from plants that are attacked by a herbivore can prime the defense of other plants for enhanced defense induction upon herbivory or they can attract natural enemies of herbivores that can act as bodyguards of the plants by ridding them of the herbivores (e.g. Dicke *et al.*, 1990; Bruin *et al.*, 1992; Karban *et al.*, 2003). The latter is also known as plant indirect defense, as it affects the herbivores indirectly by attracting predators or parasitoids that attack the attackers of the plants. Indirect plant defense has been subject to a manifold of studies. However, only by using behavioral studies in combination with molecular and chemical methods the underlying mechanisms that regulate and modulate plant defense are starting to be unraveled. Already a rapidly growing body of knowledge exists on the expression of plant defense to a single herbivore in terms of changes in plant gene transcription, metabolite biosynthesis, and arthropod behavior (e.g. Dicke and Van Loon, 2000; Kessler and Baldwin, 2002; Heide and Baldwin, 2004; De Vos *et al.*, 2005; Thompson and Goggin, 2006; Howe and Jander, 2008; Mithöfer and Boland, 2012). However, plants are subjected to a multitude of herbivores which can attack in spatially and temporally separated events. Induced herbivore-specific fluctuations in defense-related phytohormones and cross-talk between phytohormone signaling pathways, may thus significantly alter the plant phenotype and significantly change defense expression compared to single herbivory (Dicke *et al.*, 2009; Stam *et al.*, 2014). Such changes in plant phenotype can affect all members of the community associated with a plant through direct or indirect interactions (e.g. Denno *et al.*, 1995; Soler *et al.*, 2005; Soler *et al.*, 2007; De Boer *et al.*, 2008). Establishing a solid basis in the understanding of molecular and chemical plant responses to multiple herbivores can help us to comprehend how plants deal with the complexity of interactions that they are exposed to in their natural environment.

The aim of the thesis was to use a multidisciplinary approach, with focus on molecular and chemical methods, combined with behavioral investigations, to elucidate the mechanisms of plant responses to multiple herbivory that affect tritrophic interactions through phenotypic changes in plants. I addressed the following research questions:

Question I: Can plant genes and metabolites that are involved in indirect defense be primed with low doses of phytohormones?

Question II: Does minor herbivory change or prime plant genes and metabolites for enhanced induction of indirect defense by subsequent herbivory?

Question III: Do herbivores that differ in feeding mode differentially influence plant indirect

defense in terms of gene transcription and metabolites?

The tritrophic system which was used to study these research questions consists of Lima bean plants, the generalist herbivorous mite *Tetranychus urticae* and one of its natural enemies, the specialist predatory mite *Phytoseiulus persimilis*. The principal volatile compounds that affect *P. persimilis* are known and some defense gene sequences are available. Particularly, the availability of the gene sequence of *Phaseolus lunatus* β -ocimene synthase (*PIOS*), the enzyme that leads to biosynthesis of a principal attractant of *P. persimilis*, namely the monoterpene (*E*)- β -ocimene, makes it possible to study the effects of herbivory from gene to metabolite to resulting behavior.

Phytohormones and priming in plant defense

Defense signaling pathways, the major ones being the jasmonic acid (JA) pathway and the salicylic acid (SA) pathway, with JA and SA as hormonal signals, are involved in regulating the synthesis of defensive metabolites. Application of these phytohormones has been shown to effectively induce plant direct and indirect defense responses in terms of gene transcription, metabolite synthesis, and resulting arthropod behavior (Dicke *et al.*, 1999; Heidel and Baldwin, 2004; Lou *et al.*, 2005; Ozawa *et al.*, 2008). Depending on the type of herbivore these phytohormonal pathways are induced and lead to activation of distinct sets of defense genes (De Vos *et al.*, 2005). It has been suggested that when multiple herbivores attack a plant, plant defense can become affected in different manners depending on factors such as the sequence of arrival, defense pathways induced, magnitude of defense induction, and time interval between infestations (Underwood, 1998; Zhang *et al.*, 2009; Engelberth *et al.*, 2011; Erb *et al.*, 2011; Wei *et al.*, 2014). Moreover, it has been proposed that in certain situations plants are able to form a sort of memory in response to a biotic stress, such as herbivory, which enhances the plant defense response to subsequent stresses (Frost *et al.*, 2008). According to Gális *et al.* (2009) there are different mechanisms by which this memory formation might occur, that is via changes in the response time, increased signal amplitude, or baseline levels of phytohormones. In fact, due to their importance in plant defense signaling, phytohormones are likely to play a pivotal role in priming.

A role of phytohormones and priming in plant indirect defense mechanisms in a multiple herbivore context

Lima bean plants respond to exogenous application of the phytohormone JA with the activation of induced indirect defense mechanisms comprised of extrafloral nectar (EFN) excretion and volatile emission (Dicke *et al.*, 1999; Heil, 2004). In Chapter 2, I have reported on the effect of different doses of JA, SA, and infestation levels of the herbivore *T. urticae* on transcript levels of several relevant defense genes. Indeed, *PIOS* transcript levels were increased in response to JA application and *T. urticae* infestation in a dose-response and density-dependent manner. In fact, induction of indirect defense against *T. urticae* has been shown to require JA-related signaling (Dicke *et al.*, 1999; Ament *et al.*, 2004). However, *PIOS* transcript levels were decreased in a dose-dependent manner in response to SA application. Activation of plant defense genes results from the induction of the signaling pathways and

can be affected by antagonistic cross-talk (Chapter 2; Zhang *et al.*, 2009; Wei *et al.*, 2014). Consequently, antagonistic cross-talk between signaling pathways provides a regulatory mechanism at the transcriptional level to fine-tune plant defense responses (Pieterse *et al.*, 2012; Wei *et al.*, 2014).

Interestingly, exogenous application of low doses of phytohormones that do not induce plant defenses directly, can lead to enhanced induction of defense mechanisms when followed by herbivory (Gols *et al.*, 2003; Engelberth *et al.*, 2011). In Chapter 3, I investigated the underlying mechanisms of this enhanced indirect defense mediated by *P. persimilis*. Application of a low dose of JA resulted in an increase in endogenous JA levels, which could induce synergistic effects on gene transcription and metabolites when followed by herbivory by *T. urticae*. Moreover, continuous feeding by herbivores would be expected to lead to increased phytohormone levels; however, feeding by a low density of only four *T. urticae* per plant did not affect phytohormone levels. Nevertheless, herbivory by *T. urticae* might induce JA accumulation at earlier time points which is supported by observed increases in the transcript level of the JA-responsive gene *PIOS* (Chapter 3; Zhang *et al.*, 2009). Moreover, whereas 45 *T. urticae* induced transcription of enzyme genes involved in the biosynthesis of monoterpenes and diterpenes in tomato within 24 h, volatile emissions and predator attraction is only increased at day four (Kant *et al.*, 2004). Nevertheless in Lima bean, we reported priming of the monoterpene synthase *PIOS* and differences in volatile profiles already after 48 h. The emission of the predator-attracting volatile (*E*)- β -ocimene showed evidence of priming during the afternoon, but not during the morning. Consequently, temporal dynamics as well as the diurnal cycle in biosynthesis affect plant defenses significantly (Loughrin *et al.*, 1994; Underwood, 2012). In fact a study by Underwood *et al.* (2012) suggested that plant resistance against a subsequent herbivore can even change from enhanced resistance due to priming to enhanced susceptibility over time.

Gális *et al.* (2009) suggested that plant memory formation, or priming, is expressed by an increase in baseline levels of phytohormones, shorter response time to subsequent attack, or increased response amplitude after attack. However, the priming effect in Chapter 3 appeared not to be caused by an increase in baseline levels of endogenous phytohormone levels or increased response amplitude for phytohormone levels in plants with multiple bouts of defense induction compared to control plants. Because we did not further investigate the temporal dynamics of the phytohormone levels, we cannot exclude other mechanisms. However, priming can occur at different levels of biological organization as shown in Chapter 3. In fact, in Arabidopsis accumulation of mitogen-activated protein kinases (MAPKs) or inactive forms of transcription factors is related to priming (Beckers *et al.*, 2009).

A role of previous herbivory in induced (indirect) plant defense mechanisms in a multiple herbivore context

Plant defense responses can become primed in response to biotic stress caused by e.g. herbivory, oviposition, or HIPVs from neighboring plants (Arimura *et al.*, 2000; Kessler and Baldwin, 2004; Kim *et al.*, 2012; Pashalidou *et al.*, 2013). Phytohormonal signaling underlies

the induction of plant defense and it has been shown that priming of indirect defense is feasible via application of exogenous JA (Chapter 3; Gols *et al.*, 2003). However, phytohormone application does not fully resemble the defense induction by herbivory (Ozawa *et al.*, 2008). Specialist predators are able to distinguish HIPV blends emitted from phytohormone-induced plants compared to blends emitted from plants that experienced actual herbivory (Dicke *et al.*, 1999). In chapters 4, 5, and 6 I investigated whether minor herbivory by caterpillars and *T. urticae* mites can cause a priming effect in indirect defense against *T. urticae*. Caterpillars and *T. urticae* are expected to induce the JA signaling pathway and accordingly we found up-regulation of the JA-inducible *PIOS* gene in response to tissue damage by either herbivore throughout the chapters. However, previous defense induction by caterpillars or *T. urticae* did not result in enhanced or primed indirect defense on the level of gene transcription or volatile emission. Moreover, although *T. urticae* induced *PIOS* transcript levels compared to control plants in all chapters, we did not record an increase in endogenous phytohormone levels in response to mite treatment. Generally, repeated feeding by herbivores results in increases in plant defense according to the herbivore density and damage level (Underwood, 2000; Gols *et al.*, 2003; Mithöfer *et al.*, 2005). In fact, plants increase resistance in proportion to the extent of damage until approximately 90% of leaf area has been lost (Baldwin and Schmelz, 1994; Underwood, 2000). However, Ozawa *et al.* (2000) showed that an infestation by 100-150 *T. urticae* for 24 h induced defenses under control of both, the JA- and SA-related signaling pathway. SA-inducing herbivores have been shown to interfere with JA-related plant defenses (Moran *et al.*, 2002; Zhang *et al.*, 2009; Schwartzberg *et al.*, 2011; Soler *et al.*, 2012; Zhang *et al.*, 2013). In the arms race between plants and herbivores, it is hypothesized that herbivores develop mechanisms to avoid detection and suppress plant defenses. Highly polyphagous pests such as *T. urticae*, are known to adapt to and overcome constitutive and induced defenses within few generations (Agrawal, 2000; Kant *et al.*, 2008). Consequently, *T. urticae* might induce JA responses, as reflected by *PIOS* induction, but also able to quickly downregulate JA accumulation. This might be achieved by using the plant's own regulatory mechanisms, e.g. the antagonistic cross-talk between signaling pathways. After all, spider mites induce both JA and SA (Ozawa *et al.*, 2000). Memory formation might thus be more complicated against certain herbivores than others. Interestingly, caterpillar feeding and application of caterpillar oral secretions did not enhance or sensitize indirect defense mechanisms against *T. urticae* either. Investigating other time points and mechanisms may be useful in studying the plant-mediated interaction with *T. urticae*. De Vos *et al.* (2006) showed that caterpillar feeding can enhance PR- gene transcription in Arabidopsis when followed by pathogen infestation. In fact, in Chapter 4 the previous application of caterpillar oral secretions followed by an infestation by *T. urticae* results in increased levels of a gene potentially involved in plant direct defense, i.e. the pathogenesis-related protein PR 4.

Effect of herbivore feeding mode on plant defense induction

Traditionally, plant herbivores are characterized as JA or SA inducers based on their mode of feeding. Biting-chewing herbivores, such as caterpillars, and certain cell-content feeding mites are categorized as JA-signaling-pathway inducers (McConn *et al.*, 1997; Li *et al.*, 2002; Pieterse *et al.*, 2012). In contrast, piercing-sucking herbivores are categorized as SA-

signaling-pathway inducers (Kempema *et al.*, 2007). Exogenous application of the respective phytohormones has been used to induce defense responses that mainly resemble the ones induced by the corresponding herbivore feeding category (Dicke *et al.*, 1999; Zhang *et al.*, 2009). In fact, herbivorous arthropods induce phytohormonal signal signatures, whereby insects of the same or different feeding guilds induce the activation of specific sets of defense-related genes and transcriptomic changes (De Vos *et al.*, 2005; Bidart-Bouzat and Kliebenstein, 2011). Other members of the community such as natural enemies of herbivores, rely on host-location cues provided by plants, when searching for their arthropod food source. Particularly specialist carnivores are expected to be able to distinguish between volatile blends induced by specific herbivores in order to locate their respective prey or host. Consequently, especially specialist natural enemies are expected to rely on differences in volatile blends emitted from plants attacked by different herbivores and herbivore combinations. Stam *et al.* (2014) suggested that when herbivores attack a plant sequentially different effects on interactions can occur. The effects include priority effects, overriding effects, and canalization. Hereby factors such as sequence of arrival, defenses induced, amplitude of defense induction and time lag between multiple herbivores might play an important role. Attack by herbivores with different feeding modes can have beneficial or adverse effects on plant indirect defense depending on the defense signaling pathways induced (De Boer *et al.*, 2008; Zhang *et al.*, 2009).

A role of feeding mode in induced (indirect) plant defense mechanisms in a multiple herbivore context

Using molecular tools and chemical analysis there is a rapidly growing body of evidence that plants themselves are able to distinguish different herbivores and even show distinct gene activation in response to herbivores which induce the same signaling pathway(s) (De Vos *et al.*, 2005). In Chapters 4-6 we investigated whether herbivores with different feeding modes, namely caterpillars and mites, that are known to induce the same defense signaling pathway, i.e. the JA pathway, differentially affect plant indirect defense mechanisms against *T. urticae*. Zhang *et al.* (2009) showed that *T. urticae*-induced defense can be reduced by an infestation by herbivores with a different feeding mode, i.e. whiteflies, through antagonistic cross-talk of the defense signaling pathways (Zhang *et al.*, 2009). Moreover, this cross-talk induced by piercing-sucking whiteflies, also caused suppression of plant defenses against caterpillars in *Arabidopsis* (Zhang *et al.*, 2013).

We found that *PIOS* gene transcript levels did not significantly differ between plants that were exposed to multiple bouts of herbivory by herbivores with the same or different feeding mode compared to plants that experienced only one bout of herbivory. However, temporal gene transcription patterns of *PIOS* differ in response to herbivory by arthropods with different feeding mode (Chapter 5). Predatory mites, such as *P. persimilis* are able to distinguish between volatile blends of plants with prey and non-prey herbivores (Chapter 5; De Boer *et al.*, 2008) and, accordingly, we found quantitative differences in the emission rates of three principal attractants of *P. persimilis* and a green leaf volatile between plants infested by caterpillars and *T. urticae*. Moreover, we found that defense gene induction differed in

response to a subsequent exposure to *T. urticae*, depending on how damage by the caterpillar *M. brassicae* had been applied (Chapter 4). Gene transcript levels of PR-4, which codes for a chitinase and might be involved in plant direct defense, were increased in plants treated with *M. brassicae* oral secretion followed by *T. urticae* herbivory compared to plants with only *T. urticae* herbivory (Ward *et al.*, 1991). The gene's transcript levels did not show the same increase when *M. brassicae* feeding followed by *T. urticae* herbivory was compared to plants with only *T. urticae* herbivory. Whether this difference in gene induction between feeding and oral secretions also caused a shift in the temporal pattern of induction of this gene, like for caterpillar oral secretions versus *T. urticae* feeding, has not been investigated.

However, this suggests that mechanisms of plant indirect defense are indeed affected specifically by different herbivores and that plants differentiate between damage caused by feeding or mechanical damage with oral secretions by the same herbivore. Moreover, while herbivores may induce the same signaling pathway and even cause induction of the same genes, shifts in temporal patterns and amplitude of transcript levels show that plants do distinguish between those herbivores. Plants are likely to use more herbivore-specific cues besides feeding mode, such as for example herbivore-associated elicitors (HAE), and feeding rhythm to identify herbivore identity and adjust their defense responses accordingly (Mithöfer *et al.*, 2005; Bonaventure *et al.*, 2011). In fact, Bidart-Bouzzart *et al.* (2011) showed that arthropods with the same feeding mode induce different transcriptomic changes in *Arabidopsis thaliana*.

Conclusions and future perspectives

In this thesis we explored the effects of multiple herbivory on the mechanisms that guide plant indirect defense against the pest *T. urticae*. The work gives important new insights into understanding plant defense mechanisms (see Main conclusions below) and provides helpful leads for future research concerning how plants deal with multiple stresses.

Topics for future research are to investigate whether application of low doses of other phytohormones, that do not interfere with JA-related defense signaling (e.g. ethylene; see Horiuchi *et al.*, 2001) have a potential for priming of Lima bean indirect defenses and to test the applicability under field conditions. Also, the temporal dynamics of plant defense mechanisms and the effect of the diurnal cycle on priming should receive further attention. In this context it would be interesting to use a larger transcriptomic approach to see differences in priming by phytohormones versus actual herbivory. To achieve this the genome sequence of Lima bean needs to become available. In contrast to our attempt to induce priming by actual herbivory for indirect defense mechanisms, this phenomenon has been successfully observed for plant direct defense. This also means that it needs to be investigated whether the finding of the research in this thesis can be applied to other plant species and tritrophic systems. Investigating whether low-dose phytohormone application results in priming of defense mechanisms in other plant species can provide more solid knowledge about this plant defense mechanism and on what level of biological organization plant memory to stressful events is retained in different plant species. It could also give information whether plant species vary in their capability of forming memories. It would be interesting to study whether certain herbivores, such as *T. urticae* or aphids, that induce both JA and SA signaling pathways (Ozawa *et al.*, 2000; Moran and Thompson, 2001; Moran *et al.*, 2002) are able to manipulate plant resistance to avoid memory formation and thus do not lead to increased resistance against subsequent herbivores. Therefore, induction of direct defense mechanisms might also be included. Because application of caterpillar oral secretions is frequently used to mimic herbivory to induce plant defenses in a dose- and damage-controlled manner, another issue that needs to be investigated is the observed difference in defense induction between caterpillar feeding and oral secretions. For this, it would be useful to study the temporal dynamics of phytohormones and the volatile profiles of plants treated with feeding or oral secretions. This would give more information on whether our results represent temporal shifts in defense or different mechanisms being induced by these treatments.

Finally, it should be recognized that deepening our knowledge of the mechanistic aspects underlying and guiding tritrophic interactions is not only important to gain a principal understanding of complex ecological interactions. Despite the use of pesticides and other artificially introduced plant protection measures, agricultural losses due to herbivorous insects and plant pathogens, some of which can use insects as vectors, is estimated to amount to 25 % for the USA (Pimentel and Andow, 1997). At the same time, the human population keeps growing, thus increasing the demand for plant-based food and material, while the agricultural area keeps diminishing. Consequently, it remains of great importance to keep investigating the underlying mechanisms of plant defense against herbivorous arthropods to expand

fundamental knowledge that can be used to not only understand and conserve nature, but also develop tools to secure human food supply in a sustainable way.

Main Conclusions

The work described in this thesis has increased the understanding in the mechanisms that underlie Lima bean indirect defense against multiple herbivores. The main conclusions for the systems studied in this project are:

- i) Plant indirect defense mechanisms can be primed.
- ii) Priming of plant indirect defense mechanisms is not necessarily based on increased levels of phytohormones.
- iii) Different herbivores induce differential temporal dynamics in induced indirect plant defense mechanisms.
- iv) Feeding by arthropods or the combination of artificial wounding and elicitor application differ in their effects on defense gene transcription by shifting temporal patterns.
- v) Plant indirect defense mechanisms are stable to multiple herbivory by conspecific and heterospecific herbivores that induce the same defense pathway even if they differ in feeding mode.

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Summary

Plants live in complex environments and are under constant threat of being attacked by herbivores, such as insects and mites. Next to a remarkable ability to regenerate, plants possess sophisticated defense mechanisms. Two types of defenses are generally distinguished: constitutive and induced defenses. Constitutive defense includes for example thorns and trichomes (hairs). Induced defenses include chemical compounds such as toxins and herbivore-induced plant volatiles or extrafloral nectar. Induced defense can be further divided into direct and indirect mechanisms. The first directly act against the attacker by affecting its behavior or reducing its growth rate, whereas indirect defenses involve the attraction of natural enemies of herbivores that can act as a kind of bodyguards to plants. Natural enemies of herbivores use odors emitted by damaged plants that serve as a “cry for help” to find their prey or host herbivore. The odors emitted by damaged plants can consist of up to 200 compounds, of which a fraction is used by a natural enemy to find its food source, the herbivore. Nevertheless, based on the odor blend natural enemies can gain information about the identity of the plant, identity of the herbivore, developmental stage of the herbivore and so on.

Many studies have investigated the herbivore-induced changes that occur at different levels of biological organization such as plant hormone levels (phytohormones), gene level, metabolite level, and the behavior of natural enemies and herbivores in response to such changes. However, in their natural environment these interactions are much more complicated than investigated in these studies. For instance, plants are frequently attacked by more than one herbivore. This attack by multiple herbivores can occur simultaneously or in events spaced over time, on the same organ or on different organs, which can have different effects on how plant defenses are expressed. This, however, has not been investigated for many systems and different scenarios.

Recently, it has been suggested that plants even possess a sort of memory, which allows them to respond to a second attack more quickly and more strongly than when the first attack had not taken place. This phenomenon, sometimes referred to as “priming”, is often compared to mammalian vaccination, however, plants do not possess an immune system comparable to that of mammals. Nevertheless, plants do possess a complex signaling network, which allows them to sense and recognize a herbivorous attack and initiate adequate defense responses. Many studies have investigated these signaling networks and their interactions. Yet, studies addressing how multiple herbivore attacks shape defense responses and the underlying signaling networks have only been initiated relatively recently.

The aim of this thesis was therefore to use a multidisciplinary approach, with focus on molecular and chemical methods, combined with behavioral investigations, to elucidate the mechanisms of plant responses to multiple herbivory that affect a tritrophic system consisting of a plant, an herbivore and a natural enemy. In Lima bean plants the five principal components that mediate

the attraction of the predatory mite *Phytoseiulus persimilis* to plants infested with its prey, the herbivorous mite *Tetranychus urticae*, are known to consist of (*E*)-4,8-dimethylnona-1,3,7-triene [(*E*)-DMNT] and (*E,E*)-4,8,12-trimethyltrideca-1,3,7,11-tetraene [(*E,E*)-TMTT], linalool, methyl salicylate (MeSA), and (*E*)- β -ocimene. Moreover, despite the fact that the Lima bean genome has not been sequenced yet, sequences of a limited number of genes potentially involved in Lima bean defense have been identified. Since the predatory mite *P. persimilis* is a specialist predator that preys only on mites in the genus *Tetranychus*, this predator heavily relies on accurate information conveyed by plant odors.

In Chapter 1, relevant literature is reviewed about plant defense and tritrophic interactions and the study system is introduced in more detail.

In Chapter 2, I present studies on the response of three defense-related genes, *Phaseolus lunatus* lipooxygenase (*PILOX*), *Phaseolus lunatus* β -ocimene synthase (*PIOS*) and *Phaseolus lunatus* pathogenesis-related protein 4 (*PIPR-4*) to different doses of phytohormones and different densities of the herbivore *Tetranychus urticae*. Exogenous application of phytohormones was used to simulate herbivory and to induce defense gene transcription. The jasmonic acid (JA)-responsive gene *PIOS* responded to exogenous JA and salicylic acid (SA), reflecting the antagonistic interaction between the JA and SA phytohormone signaling pathways. Furthermore, *PIOS* transcript levels positively correlated to density of JA-inducing *T. urticae* mites. Transcript levels of another JA-responsive gene, *PILOX*, did not show a correlation to phytohormone doses or herbivore densities at the time point investigated here. The previously reported methyl salicylate-responsive gene, *PIPR-4*, did not respond to treatment with SA.

In Chapter 3, I report on experiments to investigate how application of a low dose of JA followed by minor herbivory by *T. urticae* spider mites affects gene transcript levels of *PIOS*, emission of (*E*)- β -ocimene and nine other plant volatiles commonly associated with herbivory. Furthermore, I investigated the plants' phytohormonal response. Application of a low dose of JA increased *PIOS* transcript levels in a synergistic manner when followed by minor herbivory for both simultaneous and sequential infestation. Emission of (*E*)- β -ocimene was also increased, and only JA, but not SA, levels were affected by treatments. Analysis of other volatiles showed overlap between volatile blends of plants exposed to different treatments. Thus, a low-dose JA application results in a synergistic effect on gene transcription and an increased emission of a volatile compound involved in indirect defense after herbivore infestation. This connects well to earlier experiments that had shown that the application of a low JA dose enhanced the attraction of *P. persimilis* when the plants were subsequently infested with spider mites.

In Chapter 4, I used feeding damage or the combination of mechanical damage and oral secretions of the caterpillar species *Mamestra brassicae* and *Spodoptera exigua* to study the damage- and species-specific effects on transcript levels of three defense-related genes in Lima bean, i.e. *PILOX*, *PIOS*, and *PIPR-4*. Since induction of defense can affect subsequent herbivores, I also investigated how the induction of defense genes by feeding damage or mechanical damage plus oral secretion affected the defense response to subsequent

herbivory by *T. urticae* spider mites. Whereas patterns of gene transcription were mostly identical in response to the two caterpillar species, feeding damage or mechanical damage plus caterpillar oral secretion caused differential induction of the transcription of defense genes. Nevertheless, compared to plants with single herbivory, plants with dual herbivory only showed differential gene induction for *PIPR-4*. Plants responded differently to caterpillar feeding than to mechanical damage plus caterpillar oral secretion, which resulted in different effects on plant direct and indirect defense against subsequent herbivores as well.

In Chapter 5, I investigated the effect of sequential induction of plant defense by *M. brassicae* caterpillar oral secretion and an infestation by *T. urticae* spider mites on the expression of indirect plant defense in Lima bean plants. The effect on indirect defense was assessed using behavioral assays with the specialist predatory mite *P. persimilis* in an olfactometer, headspace analysis of 11 major herbivore-induced plant volatiles including (*E*)- β -ocimene and transcript levels of the corresponding gene *PIOS*. Predatory mites were found to distinguish between plants induced by spider mites and plants induced by the combination of artificial mechanical damage and caterpillar oral secretion but not between plants with single spider mite infestation and plants induced by caterpillar oral secretion prior to spider mite infestation. Indeed, the volatile blends emitted by plants induced by spider mites only and the sequential induction treatment of caterpillar oral secretion followed by spider mite infestation, were similar. The data presented in this thesis suggest that the induction of plant indirect defense is not affected by previous treatment with oral secretion of *M. brassicae* caterpillars.

In Chapter 6, I studied the effect of herbivory by *T. urticae* mites on two components of induced indirect defense against subsequent herbivory by conspecific mites on Lima bean. We studied the emission of (*E*)- β -ocimene and 12 other plant volatiles commonly associated with herbivory, as well as the transcription of two genes involved in Lima bean defense, namely *PIOS* and *PIPR-4*. Volatile profiles and gene transcript level of plants attacked by herbivores differed significantly from that of control plants. Emission of volatiles did not differ between plants that experienced two bouts of herbivore attack by conspecific spider mites compared to plants that experienced only one bout of spider mite attack. Moreover, transcript levels of *PIOS* and *PIPR-4* did not differ for these treatments. The results suggest that Lima bean plants do not increase defense in response to sequential herbivory by two-spotted spider mites under the exposure regime tested.

The results are discussed in the context of recent literature in Chapter 7. In conclusion, the work compiled in this thesis presents new insights in the mechanisms of induction of indirect plant defense and tritrophic interactions in a multiple herbivore context and provides helpful leads for future research concerning how plants deal with multiple stresses.

Samenvatting

Planten leven in complexe omgevingen en staan onder de constante dreiging aangevallen te worden door plantenetende organismen zoals insecten en mijten. Naast een opmerkelijk vermogen om te regenereren, bezitten planten verfijnde verdedigingsmechanismen. In het algemeen worden twee soorten verdediging onderscheiden: constitutieve en induceerbare verdediging. Constitutieve verdediging omvat bijvoorbeeld doorns en trichomen (haren). Induceerbare verdediging is met name gebaseerd op chemische verbindingen, zoals toxines, afwerende vluchtige stoffen of extraflorale nectar. Geïnduceerde verdediging kan verder onderverdeeld worden in directe en indirecte geïnduceerde verdediging. De eerste beïnvloedt de aanvaller direct via bijvoorbeeld het veranderen van het gedrag of het verminderen van de groei, terwijl indirecte verdediging werkt via natuurlijke vijanden van planteneters. Deze natuurlijke vijanden van planteneters gebruiken geuren die door beschadigde planten verspreid worden. Het geurmengsel dat verspreid wordt door beschadigde planten kan uit enkele tientallen tot wel 200 verbindingen bestaan. Een aantal daarvan wordt gebruikt door een natuurlijke vijand van planteneters om zijn voedselbron te vinden. Niettemin kan het geurmengsel informatie bevatten over de identiteit van de plant, de identiteit van de planteneter, het ontwikkelingsstadium van de planteneter, enz.

Veranderingen die gepaard gaan met geïnduceerde verdediging zijn onderzocht op verschillende niveaus van biologische organisatie, zoals gen-niveau, metaboliet-niveau, en individu-niveau. Op het individu-niveau wordt bijvoorbeeld het gedrag van natuurlijke vijanden en planteneters bestudeerd in reactie op veranderingen in de emissie van metabolieten. In hun natuurlijke omgeving zijn deze interacties veel ingewikkelder dan onderzocht in zulke studies. Zo worden planten vaak aangevallen door meer dan één planteneter. Deze aanval door meerdere planteneters kan gelijktijdig of verspreid in de tijd plaatsvinden, op hetzelfde orgaan of op verschillende organen, wat verschillende effecten op verdedigingsmechanismen van planten kan hebben. Deze effecten op verdedigingsmechanismen zijn slechts voor enkele plantensoorten onderzocht.

Recentelijk is gesuggereerd dat planten zelfs een soort geheugen bezitten, dat hen, na een eerste aanval, in staat stelt om sneller en sterker te reageren op een tweede aanval. Dit verschijnsel, ook wel “priming” genoemd, wordt vaak vergeleken met inenten, echter planten hebben geen immuunsysteem zoals zoogdieren. Planten, daarentegen, bezitten een complex signaleringsnetwerk, waardoor ze planteneters kunnen herkennen, en hun verdediging kunnen opstarten. Veel studies hebben deze signaleringsnetwerken en hun interacties onderzocht, maar onderzoek naar verdedigingsreacties tegen meerdere planteneters en de onderliggende signalerende netwerken is schaars.

Het doel van dit proefschrift was dan ook om een multidisciplinaire benadering te volgen door gebruik te maken van moleculair-genetische en chemisch-analytische methoden, gecombineerd met gedragsonderzoek, om de invloed van meerdere planteneters op

de mechanismen van verdediging in een tritroof systeem te onderzoeken, dat bestaat uit een plant, een planteneter en een natuurlijke vijand. In Limaboon zijn de vijf belangrijkste componenten van het geurmengsel die een rol spelen in de aantrekking van de roofmijt *Phytoseiulus persimilis* nadat de planten geïnfecteerd zijn door plantenetende mijten (*Tetranychus urticae*), bekend. Deze componenten zijn (*E*)-4,8-dimethylnona-1,3,7-trieen en (*E,E*)-4,8,12-trimethyltrideca-1,3,7,11-tetraeen, linalool, methylsalicylaat en (*E*)- β -ocimeen. Bovendien zijn de sequenties van een beperkt aantal genen, die mogelijk betrokken zijn bij verdediging, geïdentificeerd. De roofmijt *P. persimilis* is een specialistische roofvijand die alleen mijten in het geslacht *Tetranychus* eet. Daarom is deze roofmijt sterk afhankelijk van nauwkeurige informatie die beschikbaar is in de vorm van plantengeuren.

In Hoofdstuk 1 wordt een overzicht gegeven van de relevante literatuur over verdediging van planten en tritrofe interacties en het studiesysteem wordt in detail geïntroduceerd.

In Hoofdstuk 2 presenteer ik het effect van behandeling van planten met verschillende doses van enkele fytohormonen en verschillende dichtheden van de planteneter *Tetranychus urticae* op drie genen die een rol spelen in de verdediging van Limaboon, *Phaseolus lunatus* lipoxxygenase (*PILOX*), *Phaseolus lunatus* β -ocimene synthase (*PIOS*) en *Phaseolus lunatus* pathogenesis-related protein 4 (*PIPR-4*). Uitwendige toediening van plantenhormonen wordt vaak gebruikt voor het simuleren van herbivorie en het induceren van de transcriptie van genen die betrokken zijn bij plantenverdediging. De transcriptie van het gen *PIOS*, dat door behandeling met het fytohormoon jasmonzuur (JA) wordt aangeschakeld, wijst op een antagonistische interactie tussen de JA- en SA-signalwegen. Bovendien was de transcriptie van *PIOS* positief gecorreleerd met herbivorie door *T. urticae* mijten. De vraat van deze mijten induceert JA in planten. De transcriptie van het andere gen dat op JA reageert, *PILOX*, liet geen correlatie zien met *T. urticae*-dichtheden of plantenhormonen op het tijdstip dat hier onderzocht is. Het salicylzuur(SA)-responsieve gen *PIPR-4* reageerde niet op de behandeling met SA.

In Hoofdstuk 3 beschrijf ik hoe de toediening van een lage dosis van JA de transcript-niveaus van het gen *PIOS* en de emissie van (*E*)- β -ocimeen en negen andere vluchtige plantenstoffen beïnvloedt als het gevolgd wordt door een infectie met een klein aantal *T. urticae* spintmijten. Verder heb ik de inductie van fytohormonen in de planten onderzocht. Toepassing van een lage dosis van JA verhoogde de transcript-niveaus van *PIOS* op een synergistische manier, wanneer dit gevolgd werd door infectie met een klein aantal *T. urticae*. Dit was zowel het geval bij simultane als bij sequentiële behandeling met JA en spintmijten. Emissie van (*E*)- β -ocimeen werd ook verhoogd, en het JA niveau, maar niet het SA niveau, werd beïnvloed door de behandelingen. Uit de analyse van andere vluchtige stoffen blijkt dat de samenstelling van de geurmengsels van planten met verschillende behandelingen overlapt. Zo heeft een lage dosis JA een synergistisch effect op gen-transcriptie en een verhoogde emissie van een vluchtige verbinding die betrokken is bij indirecte verdediging na besmetting door spintmijten. Dit sluit goed aan op eerdere experimenten, die hadden aangetoond dat de toepassing van een lage dosis JA in combinatie met een aantasting door spintmijten de aantrekking van *P. persimilis* versterkt.

In Hoofdstuk 4 gebruikte ik rupsenvraat of de combinatie van mechanische schade en rupsenspuug van twee rupsensoorten, *Mamestra brassicae* en *Spodoptera exigua*, om de effecten op transcript-niveaus van drie verdedigings-genen, *PILOX*, *PLOS*, *PIPR-4*, te bestuderen. Inductie van verdediging tegen een eerste aanvaller kan de verdediging tegen latere planteneters beïnvloeden. Daarom heb ik ook onderzocht hoe de inductie van genen door vraatschade of door de combinatie van mechanische schade plus rupsenspuug de verdediging in reactie op een secundaire infectie met *T. urticae* spintmijten beïnvloedt. Terwijl de patronen van gentranscriptie meestal identiek waren in reactie op vraat door de twee soorten rupsen, veroorzaakten mechanische beschadiging in combinatie met rupsenspuug differentiële inductie van de transcriptie van verdedigings-genen. In vergelijking met planten met enkelvoudige herbivorie, vertoonden planten met dubbele herbivorie alleen differentiële inductie van het gen *PIPR-4*. Planten reageerden dus verschillend op rupsenvraat en op mechanische beschadiging in combinatie met rupsenspuug, wat ook resulteert in verschillende effecten op directe en indirecte verdediging van de planten tegen latere planteneters.

In Hoofdstuk 5 beschrijf ik onderzoek naar het effect van opeenvolgende inductie van verdediging door *M. brassicae* spuug gevolgd door vraat door *T. urticae* spintmijten op de expressie van indirecte verdediging in Limaboonplanten. Het effect op de indirecte verdediging werd beoordeeld met behulp van gedragsobservaties van de specialistische roofmijt *P. persimilis* in een olfactometer, headspace analyse van 11 vluchtige plantenstoffen zoals (*E*)- β -ocimeen en de transcriptie van het *PIOS* gen. Roofmijten bleken onderscheid te maken tussen de geuren van planten geïnduceerd door spintmijten en de geuren van planten geïnduceerd door mechanische beschadiging in combinatie met rupsenspuug, maar niet tussen de geuren van planten met enkelvoudige spintmijt-infectie en planten geïnduceerd door rupsenspuug gevolgd door spintmijtvraat. Chemische analyse liet zien dat de geurmengsels afkomstig van planten met spintmijtvraat en van planten behandeld met rupsenspuug gevolgd door spintmijtvraat een vergelijkbare samenstelling hadden. De inductie van verdediging in Limaboonplanten werd niet beïnvloed door eerdere behandeling met spuug van *M. brassicae* rupsen.

Hoofdstuk 6 beschrijft de studie van het effect van spintmijtvraat op de expressie van twee componenten van indirecte verdediging en op een latere aanval door soortgenoten. Ik bestudeerde de emissie van (*E*)- β -ocimeen en 12 andere vluchtige stoffen geassocieerd met spintvraat, alsmede de transcriptie van twee genen, namelijk *PIOS* en *PIPR-4*. Geuremissie en gentranscriptie van planten met spintvraat verschilde significant van die van controle planten. Geuremissie verschilde niet tussen planten die twee aanvallen van mijten hadden ervaren in vergelijking met planten die slechts één aanval van mijten hadden ervaren. Bovendien waren transcript-niveaus van *PIOS* en *PIPR-4* niet verschillend voor deze behandelingen. De resultaten suggereren dat verdediging in Limaboon niet toeneemt in reactie op sequentiële aanvallen door spintmijten.

Tenslotte worden de resultaten besproken in de context van recente literatuur in Hoofdstuk 7. Samenvattend, het onderzoek gebundeld in dit proefschrift presenteert nieuwe inzichten in de mechanismen van inductie van indirecte verdediging van planten en tritrofe interacties in

de context van meer dan één planteneter en biedt perspectieven voor toekomstig onderzoek naar de manier waarop planten omgaan met combinaties van planteneters.

Acknowledgements

Receiving a PhD degree is so much more than a logical consequence of a series of correct choices and actions. It is not simply the doing or work of one person alone. Being able to do a PhD is mainly a privilege that is made possible by hard work in combination with support from a strong support system consisting of many people. The further I progressed with my PhD project and with increasing complexity of my project, the more I came to realize that I wouldn't be where I am today without some major support from the wonderful people around me. Some of them I would like to acknowledge here. Unfortunately, certain people are not able to read this because they have recently passed away or are too young to read, but nevertheless they need to be mentioned.

To start with, I would like to thank my closer family: Mona and Decio, Jascha and Jule (and the three kids), Jürgen and Pia. Without your support, encouragement, and trust in my abilities, I would not even be close to where I am today. You are indeed the pillars that hold me up and keep me going. You all live in my heart where I hold you close no matter how far we might be apart. I know I can always come home to you when the world knocks me down, just so I can head back out swinging.

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in the end of writing my thesis, which I greatly enjoyed. Joop, thank you especially for your understanding and kindness during rough times.

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Dr. Jenny de Jonge for being a neighbor, a colleague, cat sitter, and so much more, but especially a loyal friend since our MSc studies in Wageningen. You are dearly missed here.

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Last but not least my daughter Nynke L. Imhausen, my Minime, who drives me crazy and makes me whole again when I struggle with (PhD-)life. You are the love of my life. Your smile and your little kisses are the reason why I want to keep waking up every morning.

This is just to mention the few people that helped me during the last few years to grow as a person and researcher. Since life is a journey this can be no more but a little snapshot of who I should all thank for being here and succeeding at what I do.

Curriculum vitae



Tila Romina Menzel was born on April 24th, 1984 in Berlin, Germany. In 2001 she obtained her American highschool diploma during her 1-year stay in the state of Oregon as a foreign exchange student. Afterwards she went back to Germany to complete her education there and obtain the German highschool diploma in 2003. During her highschool time she found her passion for travelling and biology.

She went on to study in Italy in order to pursue an International first level degree in Job Creation Oriented Biotechnology, which has been established by 12 European universities with the objective to create a common mentality in students coming from universities of different countries. During her studies she travelled to different countries and gained practical experience by working in different laboratories across the world. She did an internship at the Centre d'Estudis Avançats de Blanes (Planes, Spain) where she worked with algae and nutrient-diffusing substrata, another internship at the Institut für Getreideverarbeitung (IGV) (Potsdam, Germany) where she investigated disruption methods for different algae cells to facilitate protease extraction. Finally she did a bachelor thesis project at Oregon State University (Corvallis, OR, USA) studying errantivirus sequences in insect cell lines. During an inspiring course of Plant Biotechnology taught by Prof. Dr F. Veronesi she finally decided to focus her studies on plants.

In 2010 she obtained her MSc degree at Wageningen University (Wageningen, The Netherlands) in Plant Biotechnology with emphasis on Molecular Plant Breeding and Pathology. Also during her MSc studies she participated in applied research within the Department of Plant Breeding and Bioscience, the latter in collaboration with the Department of Entomology. She worked on her PhD project from 2010-2014 under supervision of Prof. Dr M. Dicke and Prof. Dr J.J.A van Loon to study the induction of indirect plant defense in the context of multiple herbivory. During the first two years of her PhD trajet, she was a member of the PhD council of the Experimental Plant Sciences (EPS) graduate school, where she was particularly involved in organizing events such as the ExPeCtationS career day and other social events for PhD students.

List of publications

Menzel TR, Huang T-Y, Weldegergis BT, Gols R, Van Loon JJA, Dicke M. 2014. Effect of sequential induction by *Mamestra brassicae* L. and *Tetranychus urticae* Koch on Lima bean plant indirect defense. *Journal of Chemical Ecology* (in press), DOI 10.1007/s10886 - 014 - 0499 - 9.

Menzel TR, Weldegergis BT, David A, Boland W, Gols R, Van Loon JJA, Dicke M. 2014. Synergism in the effect of prior jasmonic acid application on herbivore-induced volatile emission by Lima bean plants: transcription of a monoterpene synthase gene and volatile emission. *Journal of Experimental Botany* 65, 4821 - 4831.

Wei J, Van Loon JJA, Gols R, **Menzel TR**, Li N, Kang L, Dicke M. 2014. Reciprocal antagonism between two defense-signaling pathways in Lima bean modulates volatile emission and herbivore behavior. *Journal of Experimental Botany* 65, 3289 – 3298.

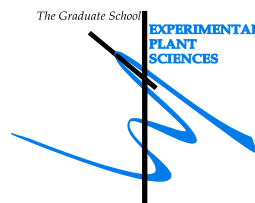
Ramirez AM, Stoop G, **Menzel TR**, Gols R, Bouwmeester HJ, Dicke M, Jongsma MA. 2012) Bidirectional secretions from glandular trichomes of pyrethrum enable immunization of seedlings. *Plant Cell* 24, 4252 – 4265.

Wang Y, van Kronenburg B, **Menzel T**, Maliepaard C, Shen X, Krens F. 2012. Regeneration and Agrobacterium-mediated transformation of multiple lily cultivars. *Plant Cell, Tissue and Organ Culture* 111, 113 – 122.

Menzel T, Rohrmann GF. 2008. Diversity of errantivirus (retrovirus) sequences in two cell lines used for baculovirus expression, *Spodoptera frugiperda* and *Trichoplusia ni*. *Virus Genes* 36., 583 – 586.

Education Statement of the Graduate School

Experimental Plant Sciences



Issued to: Tila Romina Menzel

Date: 19 November 2014

Group: Entomology

University: Wageningen University & Research Centre

1) Start-up phase	<u>date</u>
► First presentation of your project	
Priming in indirect defenses of Lima bean plants against herbivorous arthropods	Feb 19, 2010
► Writing or rewriting a project proposal	
► Writing a review or book chapter	
► MSc courses	
Insect-Plant Interactions (ENT-50806)	Apr 26- May 27, 2010
► Laboratory use of isotopes	
<i>Subtotal Start-up Phase</i>	
<i>7.5 credits*</i>	
2) Scientific Exposure	<u>date</u>
► EPS PhD student days	
PhD student day, Utrecht University	Jun 01, 2010
PhD student day, Wageningen University	May 20, 2011
► EPS theme symposia	
EPS symposium Theme 2, 'Interactions between plants and biotic agents', Leiden University	Jan 15, 2010
EPS symposium Theme 2, 'Interactions between plants and biotic agents', Wageningen University	Feb 03, 2011
EPS symposium Theme 2, 'Interactions between plants and biotic agents', Utrecht University	Feb 10, 2012
► NWO Lunteren days and other National Platforms	
NWO-ALW meeting 'Experimental Plant Sciences', Lunteren, NL	Apr 19-20, 2010
NWO-ALW meeting 'Experimental Plant Sciences', Lunteren, NL	Apr 04-05, 2011
NWO-ALW meeting 'Experimental Plant Sciences', Lunteren, NL	Apr 14-15, 2014
Entomologendag (NEV)	Dec 16, 2010
Entomologendag (NEV)	Dec 13, 2013
► Seminars (series), workshops and symposia	
Invited seminars at Entomology	2010-2014
Invited seminars at department PSG	2010-2014
5th Plant-Insect Interactions workshop, Wageningen University	Nov 11, 2010
ExPectationS (EPS career day)	Nov 19, 2010
ExPectationS (EPS career day)	Nov 18, 2011
8th Plant-Insect Interactions workshop, Wageningen University	Oct 24, 2013

CONTINUED ON NEXT PAGE

<p>► Seminar plus</p> <p>Invited lecturer Georg Jander, Boyce Thompson Institute for Plant Research, USA</p> <p>International symposia and congresses (highly recommended)</p> <p>14th International Symposium on Insect-Plant interactions (SIP-14), Wageningen, NL</p> <p>► Presentations</p> <p>Poster: Transcriptional response of Lima bean plants to herbivory after low dose phytohormone application (SIP-14; Poster)</p> <p>Presentation: Transcriptional response of Lima bean plants to herbivory after low dose phytohormone application (Summerschool Environmental Signaling)</p> <p>Poster: Effect of heterospecific herbivores on biological pest control (EuroVOL Summer School)</p> <p>Presentation: The effects of sequential infestation by herbivores on the response of the predator <i>Phytoseiulus perimilis</i> (Entomologendag 2013)</p> <p>► IAB interview</p> <p>Meeting with a member of the International Advisory Board of EPS</p> <p>► Excursions</p> <p>Keygene excursion</p>	<p>Jan 10, 2011</p> <p>Aug 13-18, 2011</p> <p>Aug 13-18, 2011</p> <p>Aug 22-24, 2011</p> <p>Sep 09, 2013</p> <p>Dec 13, 2013</p> <p>Nov 15, 2012</p> <p>Jan 26, 2012</p>
<p style="text-align: right;"><i>Subtotal Scientific Exposure</i></p>	
<p style="text-align: right;"><i>14.0 credits*</i></p>	

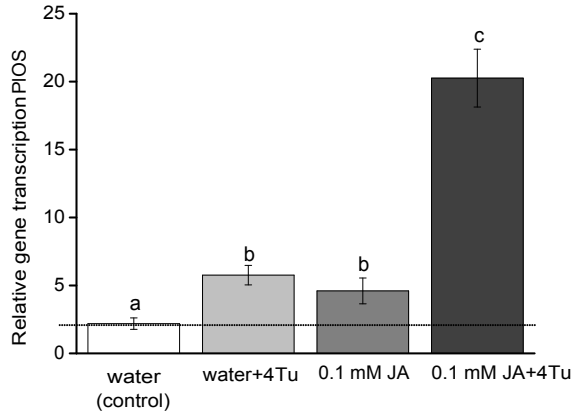
<p>3) In-Depth Studies</p> <p>► EPS courses or other PhD courses</p> <p>Postgraduate course 'Generalized linear models'</p> <p>Utrecht Summer School of Environmental Signaling</p> <p>Postgraduate course 'Advanced Statistics: Design of Experiments'</p> <p>EuroVOL Summer School</p> <p>► Journal club</p> <p>Entomology (IPI) journal club</p> <p>Entomology PhD lunches</p> <p>Priming group</p> <p>► Individual research training</p>	<p><u>date</u></p> <p>Jun 28-29, 2010</p> <p>Aug 22-24, 2011</p> <p>Oct 10-12, 2012</p> <p>Sep 09-12, 2013</p> <p>2010-2014</p> <p>2010-2014</p> <p>2010-2014</p>
<p style="text-align: right;"><i>Subtotal In-Depth Studies</i></p>	
<p style="text-align: right;"><i>7.5 credits*</i></p>	

<p>4) Personal development</p> <p>► Skill training courses</p> <p>Scientific writing/Techniques for writing and presenting a scientific paper</p> <p>Project and Time Management</p> <p>PhD competence assessment</p> <p>► Organisation of PhD students day, course or conference</p> <p>ExPeCtationS (EPS career day)</p> <p>EPS social event: PhD movie</p> <p>► Membership of Board, Committee or PhD council</p> <p>member of the EPS PhD student council</p>	<p><u>date</u></p> <p>Feb 14-17, 2012</p> <p>Nov-Dec 2011</p> <p>May-Jun.2010</p> <p>Nov 19. 2010</p> <p>Dec 14, 2011</p> <p>Jan. 2010-2012</p>
<p style="text-align: right;"><i>Subtotal Personal Development</i></p>	
<p style="text-align: right;"><i>7.4 credits*</i></p>	

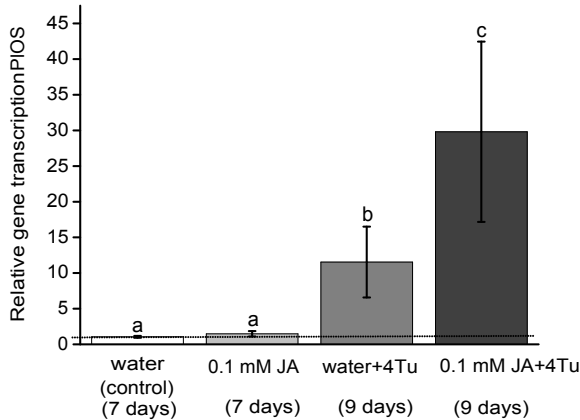
<p style="text-align: center;">TOTAL NUMBER OF CREDIT POINTS*</p>	<p style="text-align: center;">36.4</p>
<p>Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS which comprises of a minimum total of 30 ECTS credits</p> <p><i>* A credit represents a normative study load of 28 hours of study.</i></p>	

Supplemental data

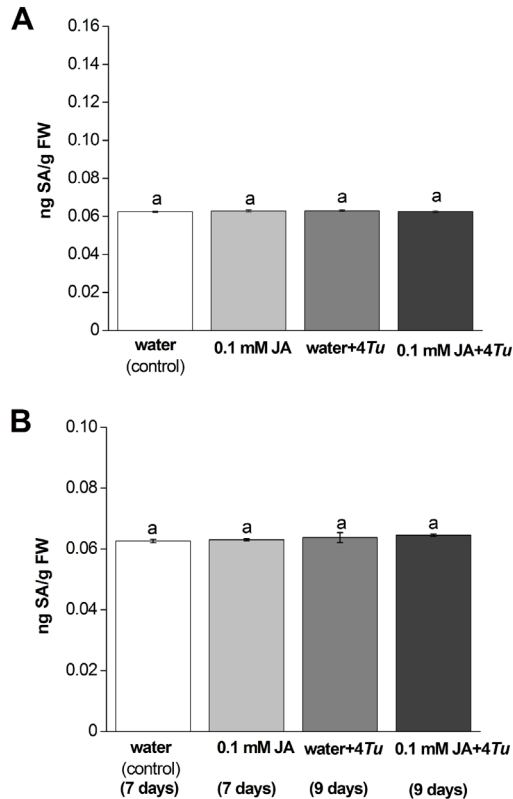
Supplemental data Chapter 3



Supplemental Figure 1. Relative gene transcript levels of *PIOS* of three independent experiments spaced in time, quantified in *P. lunatus* plants treated with i) water (control), ii) 0.1 mM JA, iii) four *T. urticae* (water + 4*Tu*), or iv) 0.1 mM JA with four *T. urticae* mites (0.1 mM JA + 4*Tu*). Simultaneous application of four *T. urticae* on plants for 48 h. Values are the mean (\pm SE) of ten to twelve biological replicates, different letters above bars indicate significant differences in transcript levels between treatments (Fisher's LSD tests, $\alpha = 0.05$). *PIOS* transcript levels were normalized to the normalization factor obtained from geometrically averaging the Ct values of the two reference genes *PIACT1* and *PINMP1* for each sample. Baseline represents transcript level in control plants.

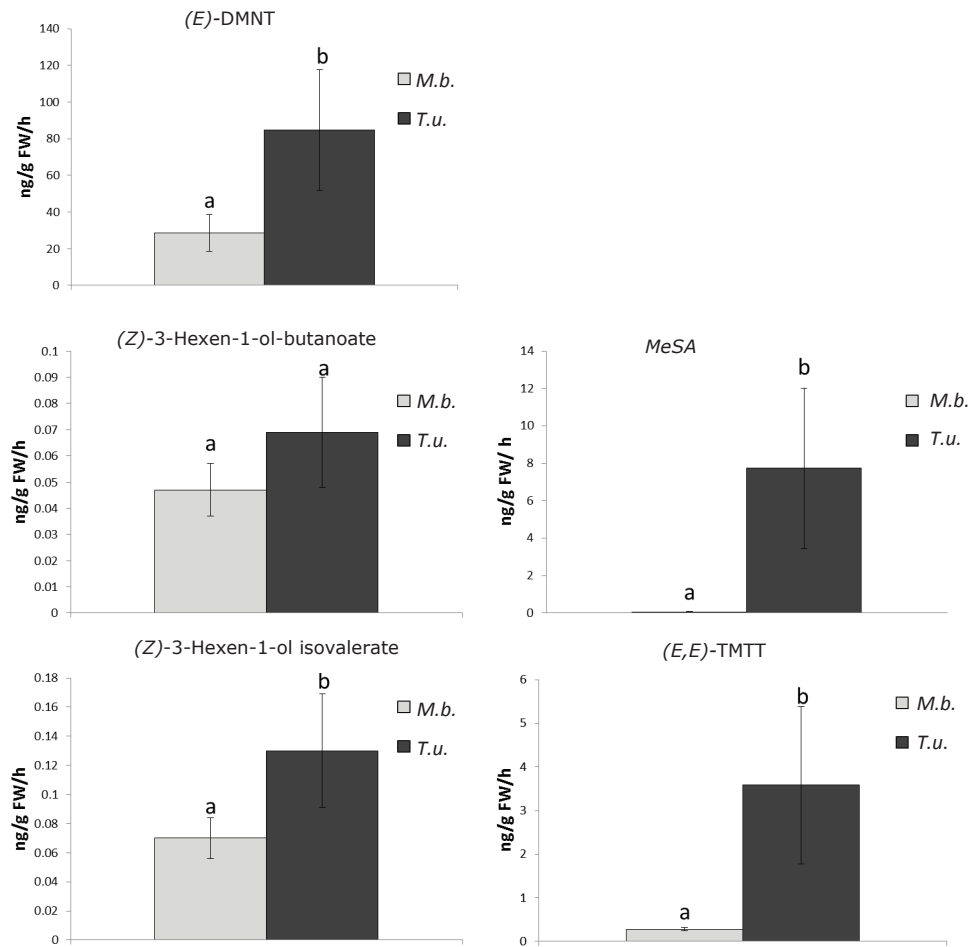


Supplemental Figure 2. Relative gene transcript levels of *PIOS* of two experiments spaced in time, quantified in *P. lunatus* plants treated with i) water (control), ii) 0.1 mM JA, iii) four *T. urticae* (water + 4*Tu*), or iv) 0.1 mM JA with four *T. urticae* mites (0.1 mM JA + 4*Tu*). Sequential application of four *T. urticae* placed on plants for 48 h after prior application with water or 0.1 mM JA seven days before. Values are the mean (\pm SE) of six to eight biological replicates, different letters above bars indicate significant differences in transcript levels between treatments (Fisher's LSD tests, $\alpha = 0.05$). *PIOS* transcript levels were normalized to the normalization factor obtained from geometrically averaging the Ct values of the two reference genes *PIACT1* and *PINMP1* for each sample. Baseline represents transcript level in control plants.

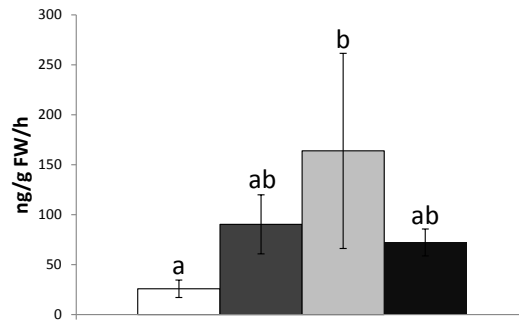


Supplemental Figure 3. SA levels in ng SA/g FW in *P. lunatus* plants treated with i) water (control), ii) 0.1 mM JA, iii) four *T. urticae* (water + 4*Tu*), or iv) 0.1 mM JA with four *T. urticae* mites (0.1 mM JA + 4*Tu*). (A) Inoculation of four adult female *T. urticae* on plants was done immediately following JA-treatment and mites had since been feeding for 48 h, and (B) inoculation of four adult female *T. urticae* for 48 h was done seven days after incubation with water or 0.1 mM JA started and mites had since been feeding for 48 h. Values are the mean (\pm SE) of four biological replicates, and were analyzed by ANOVA (A) or Kruskal-Wallis test (B) respectively ($\alpha = 0.05$).

Supplemental data Chapter 5

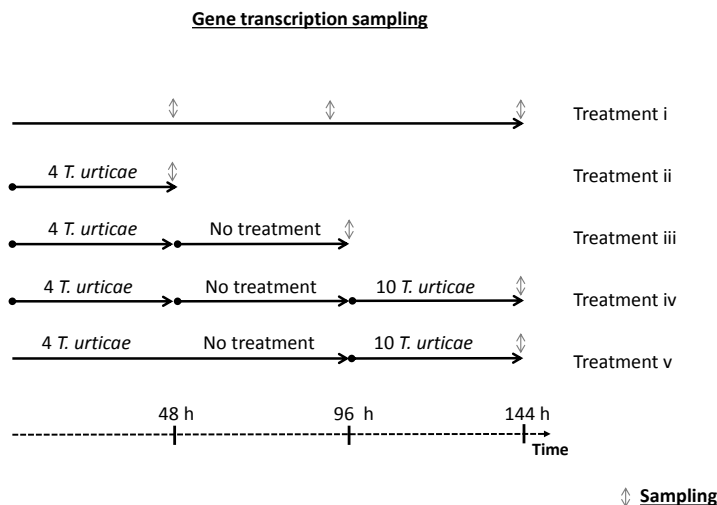


Supplemental Figure S1. Pairwise comparisons between average emission rates of volatiles showing high VIP values in plants treated with 20 *T. urticae* (*T.u.*) for 48 h or caterpillar oral secretion (*M.b.*) for 48 h. Values are the mean (\pm SE) of eight biological replicates. Different letters above bars indicate significant differences in emission rates between treatments (Mann Whitney U test, $\alpha = 0.05$).

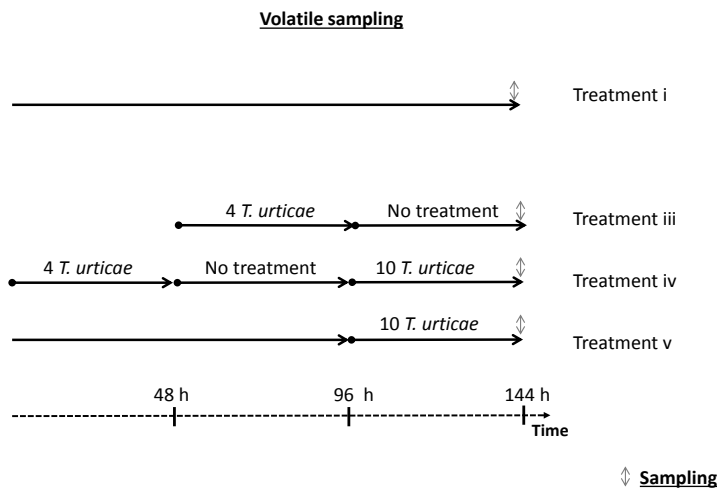


Supplemental Figure S2. Average emission rates of (*E*)- β -ocimene in clean *P. lunatus* plants or/and treated with 20 *T. urticae* for 48 h (*T.u.*) or caterpillar oral secretion [*M.b.* (48 h)] for 48 h or the combination of *M. brassicae* oral secretion for 48 h followed by infestation with 20 *T. urticae* for 48 h (*M.b.+T.u.*). Values are the mean (\pm SE) of eight biological replicates. Different letters above bars indicate significant differences in emission rates between treatments (ANOVA, Tukey's HSD tests, $\alpha=0.05$).

Supplemental data Chapter 6



Suppl. Fig. 1. Treatments and sampling for gene transcription analysis. Treatments consisted of i) control, ii) four *T. urticae*, iii) four *T. urticae* + no treatment, iv) four *T. urticae* followed by no treatment and then 10 *T. urticae*, and v) 10 *T. urticae*.

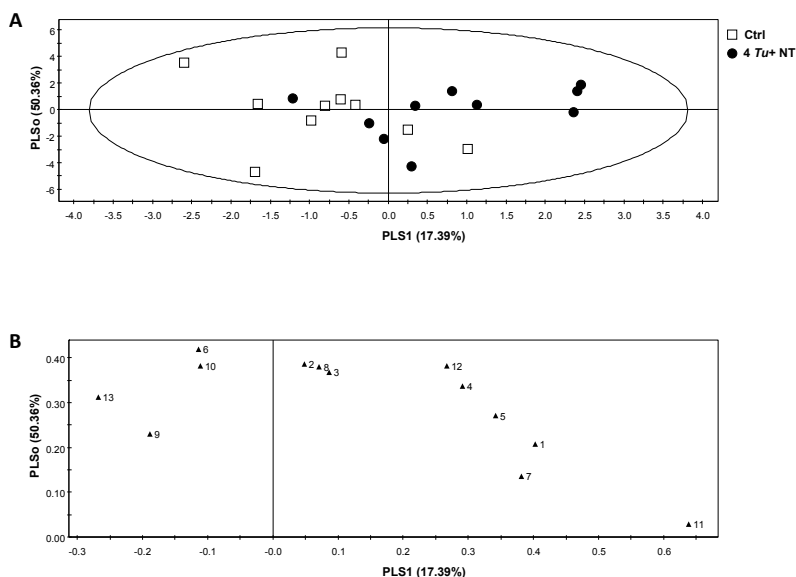


Suppl. Fig. 2. Treatments and sampling of samples for volatile sampling. Treatments consisted of i) control, iii) four *T. urticae* followed by no treatment, iv) four *T. urticae* followed by no treatment and then 10 *T. urticae*, and v) 10 *T. urticae*.

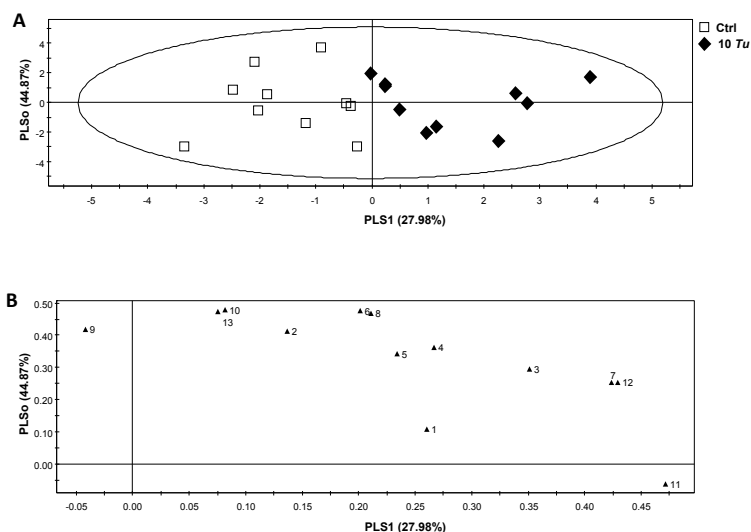
Table 1 Volatile emissions^a by Lima bean plants of the four different treatments with one-way ANOVA results of single volatile analysis and VIP values.

ID ^b	Volatile compound	Treatment				P-value ^c	VIP value
		Control (n= 10)	4 T.u. + Nothing (n= 10)	4 T.urticae + Nothing+ 10 T. urticae (n= 10)	10 T.urticae (n= 10)		
1	(E)-2-hexenal	0.03±0.01	0.05±0.01	0.04±0.01	0.04±0.01 ⁹	> 0.05	0.44
2	(Z)-3-hexen-1-ol	0.19±0.03	0.24±0.02	0.25±0.06	0.21±0.03	> 0.05	0.48
3	(Z)-3-hexen-1-ol, acetate	0.23±0.04	0.33±0.08	0.69±0.12	0.66±0.15	< 0.001	1.9
4	(E)-β-ocimene	27.14±5.43	36.28±12.52	30.96±3.08	40.09±5.58	> 0.05	0.9
5	linalool	0.22±0.05	0.29±0.03	0.25±0.05	0.28±0.03	> 0.05	0.48
6	(E)-DMNT	33.28±6.93	28.1±2.83	39.68±6.00	42.04±6.69	> 0.05	0.9
7	alloocimene	0.07±0.00 ⁹	0.09±0.01 ⁸	0.08±0.00 ⁹	0.08±0.00	> 0.05	0.71
8	(Z)-3-hexen-1-ol, butanoate	0.01±0.00 ⁹	0.01±0.00	0.02±0.00	0.02±0.00	> 0.05	0.92
9	methyl salicylate	4.11±2.32	2.35±0.69	3.93±1.61	3.57±1.38	> 0.05	0.03
10	(Z)-3-hexen-1-olisovalerate	0.05±0.01	0.04±0.00	0.05±0.01	0.05±0.01	> 0.05	0.46
11	indole	0.03±0.00	0.04±0.01	0.05±0.01	0.06±0.01	≤ 0.05	1.41
12	β-caryophyllene	0.08±0.01	0.09±0.01	0.11±0.01	0.14±0.02	≤ 0.001	1.78
13	(E-E)-TMTT	1.61±0.54	1±0.19	1.74±0.40	1.54±0.32	> 0.05	0.59

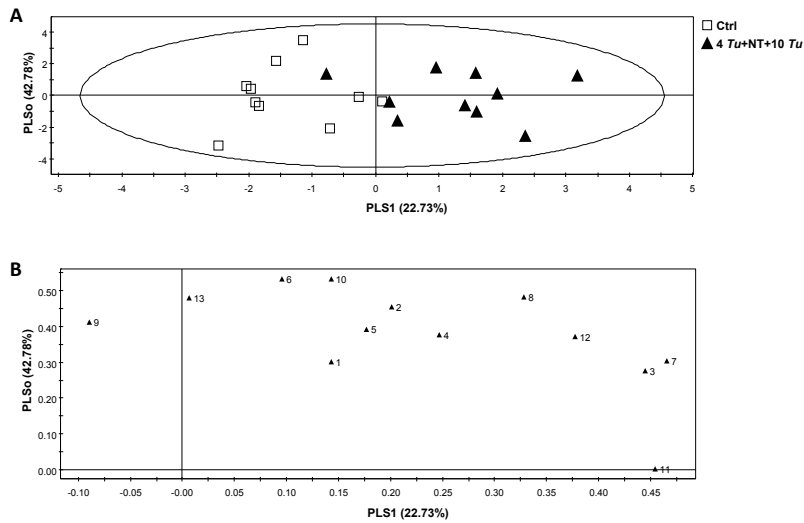
^a Volatile emissions are given as mean peak area ± SE/g fresh weight of foliage with the number of samples between brackets^b ID corresponds with the numbers presented in Fig. 1^c Numbers in superscript following emission quantities give the number of samples in which the compound was detected, if it was not found in all the samples of that treatment^c Underlined P-values indicate significant differences in emission among treatments of a compound (one-way ANOVA)



Supplemental Figure 3. Multivariate data analysis by orthogonal PLS-DA (OPLS-DA; panel A) and corresponding loading plot (panel B) of volatile blends of *P. lunatus* plants with no treatment (Ctrl), or treatment with 4 *T. urticae* for 48 h + Nothing for 48 h (4 Tu+NT). The first two principal components are given (panel A) with the percentage of variation explained in parentheses. Numbers in the loading plot (panel B) represent 1) (*E*)-2-hexenal, 2) (*Z*)-3-hexen-1-ol, 3) (*Z*)-3-hexen-1-ol, acetate, 4) (*E*)- β -ocimene, 5) linalool, 6) (*E*)-4,8-dimethylnona-1,3,7-triene [(*E*)-DMNT], 7) alloocimene, 8) (*Z*)-3-hexen-1-ol, butanoate, 9) methyl salicylate, 10) (*Z*)-3-hexen-1-ol, isovalerate, 11) indole, 12) β -caryophyllene, and 13) (*E,E*)-4,8,12-trimethyltrideca-1,3,7,11-tetraene [(*E,E*)-TMTT].



Supplemental Figure 4. Multivariate data analysis by orthogonal PLS-DA (OPLS-DA; panel A) and corresponding loading plot (panel B) of volatile blends of *P. lunatus* plants with no treatment (Ctrl), or treatment with 10 *T. urticae* for 48 h (10 Tu). The first two principal components are given (panel A) with the percentage of variation explained in parentheses. Numbers in the loading plot (panel B) represent 1) (*E*)-2-hexenal, 2) (*Z*)-3-hexen-1-ol, 3) (*Z*)-3-hexen-1-ol, acetate, 4) (*E*)- β -ocimene, 5) linalool, 6) (*E*)-4,8-dimethylnona-1,3,7-triene [(*E*)-DMNT], 7) alloocimene, 8) (*Z*)-3-hexen-1-ol, butanoate, 9) methyl salicylate, 10) (*Z*)-3-hexen-1-ol, isovalerate, 11) indole, 12) β -caryophyllene, and 13) (*E,E*)-4,8,12-trimethyltrideca-1,3,7,11-tetraene [(*E,E*)-TMTT].



Supplemental Figure 5. Multivariate data analysis by orthogonal PLS-DA (OPLS-DA; panel A) and corresponding loading plot (panel B) of volatile blends of *P. lunatus* plants with i) no treatment (Ctrl), or treatment with four *T. urticae* for 48 h followed by no treatment for 48 h and then 10 *T. urticae* for 48 h (4 Tu+ NT +10 Tu). The first two principal components are given (panel A) with the percentage of variation explained in parentheses. Numbers in the loading plot (panel B) represent 1) (*E*)-2-hexenal, 2) (*Z*)-3-hexen-1-ol, 3) (*Z*)-3-hexen-1-ol, acetate, 4) (*E*)- β -ocimene, 5) linalool, 6) (*E*)-4,8-dimethylnona-1,3,7-triene [(*E*)-DMNT], 7) alloocimene, 8) (*Z*)-3-hexen-1-ol, butanoate, 9) methyl salicylate, 10) (*Z*)-3-hexen-1-ol, isovalerate, 11) indole, 12) β -caryophyllene, and 13) (*E,E*)-4,8,12-trimethyltrideca-1,3,7,11-tetraene [(*E,E*)-TMTT].

This work was performed at the Laboratory of Entomology, Wageningen University.

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